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REMARKS

In the Advisory Action, the Examiner maintains the rejection of Claims 9-10 under 35 U.S.C. § 103 as being unpatentable over Slavin et al in view of Ildstad et al and Zhang et al.

Specifically, the Examiner contends that Applicants have argued the references individually, and not their combination.

Contrary to the Examiner's contention, Applicants have clearly argued that there is no motivation to combine the cited references to achieve the present invention.

As discussed in the Response After Final filed February 20, 2004, Slavin et al teaches that total body radiation (TBI) is not preferred, and, contrary to the Examiner's contention, does not teach "transplanting of [an] organ into [a] recipient occurs within the same day", i.e., "(a one-day protocol)" or an "engraftment rate of 100%", i.e., 100% survival rate, that can be achieved when employing TBI. The portions of Slavin et al that the Examiner relies upon do not relate to TBI, but, rather, relate to total lymphoid irradiation (TLI) which is fundamentally different from TBI.

When TBI was used, Slavin et al teaches that a 100% survival rate was not obtained (see Examples 10 and 11, and Figures 4 and 7 thereof). The Examiner is requested to note that the TBI condition employed in the examples of Slavin et al was 4.0 Gy, i.e., outside of the claimed range of at least 6.5 Gy. Thus, Slavin et al implicitly teaches that a dose of greater than 4.0 Gy is harmful, thereby teaching away from the present invention. Nowhere does Slavin et al teach that a 100% survival rate (as claimed in the present invention) can be obtained with a low dose (4.0 Gy + intravenous administration (I.V.)). That is, it is

clear from Figures 4 and 7 of Slavin et al, that the survival rate achieved therein was no higher than 80%. These results are consistent with those in Figure 2 of the present application where Group IV (6.0 Gy + portal venous administration (P.V.) or I.V.) does not give rise to a 100% survival rate.

Thus, from a combination of the teachings in Slavin et al and Ildstad et al, one would not employ TBI to achieve a 100% survival rate, i.e., one would rather employ TLI. Ildstad et al does not provide any motivation to use TBI in the method of Slavin et al particularly, when Slavin et al teaches that TBI is not preferable (see column 8, lines 63-65 thereof). There is no recognition in either Slavin et al or Ildstad et al that TBI would be advantageous and provide an unexpected advantage in the method of Slavin et al. The Examiner has apparently overlooked Applicants' arguments as to why there is no motivation to combine the teachings of the cited references to achieve the present invention. Thus, the Examiner has failed to establish a *prima facie* case of obviousness.

Moreover, as previously noted by Applicants, Ildstad et al relates to a technique using mixed chimerism, and is fundamentally different from the present invention, which uses fully allogenic chimerism.

Ildstad et al discloses that "allogenic engraftment was reliably achieved in 100%" by employing 7.0 Gy + I.V. (see Figure 1 and column 17, lines 15-16 in Ildstad et al). However, this merely indicates that the 100% of the animals conditioned by 7.0 Gy + I.V. exhibited mixed chimerism. This is clear form the expression "% recipients with chimerism" for

the vertical axis in Figure 1, and the description in column 17, lines 9-15 thereof.

This is entirely different from the effects of the present invention, i.e., "transplanting an organ into said recipient, to thereby achieve an engraftment rate of 100%".

Contrary to the Examiner's contention in the Final Office Action dated July 29, 2002, Figure 7 of Ildstad et al does not show a 100% acceptance of skin grafts after 30 days; the only skin grafts showing no rejection after 30 days are (unsurprisingly) those of the recipients themselves. On the other hand, donor-specific grafts, were already approximately 10% rejected after 20 days.

This is to be expected since, as disclosed in Ikebukuro et al, *Transplantation*, 73(4):512-518 (2002) (a copy of which is attached hereto) and Hayashi et al, *Stem Cells*, 18:273-280 (2000) (of record), when a mixed chimerism technique is employed, donor-derived BMCs gradually decrease, leading to eventually organ rejection (see the Declaration under 37 C.F.R. § 1.132 of Susuma Ikehara attached hereto).

The Examiner relies on his assertion that Ildstad et al teaches 100% skin graft acceptance after 30 days to support his position that 100% engraftment of BMCs in Ildstad et al is equivalent to a 100% organ engraftment rate in the present invention, and therefore contends that it is proper to combine Ildstad et al with Slavin et al.

However, because a skilled person in the art would note, as per Figure 7, that a 100% engraftment rate for BMCs in Ildstad et al does not lead to 100% organ engraftment, the skilled person would not be motivated to combine Ildstad et al with Slavin et al.

As described above, it can not be expected to achieve the effect essential to the present invention, i.e., the transplanted organ survives for a long time and fulfils its function, by employing the technique disclosed in Ildstad et al. Furthermore, there is no description or data in Ildstad et al from which a skilled artisan could predict or expect such an effect.

While Applicants submit that the Examiner has failed to establish a *prima facie* case of obviousness, even assuming *arguendo* that such *prima facie* case of obviousness has been raised, the evidence of record is clearly sufficient to rebut the same.

The Examiner has apparently overlooked the data in the specification (see Figure 2). As shown in Figure 2, the recipient mice given an irradiation dosage of 7 Gy in the P.V. administration group showed an engraftment rate of 100% after transplantation. This is in contrast with the recipient mice exposed to an irradiation dosage of 6.0 Gy in which the skin graft was invariably rejected within 3 weeks after transplantation. In recipient mice given an irradiation dosage of 6.5 Gy, the skin graft was rejected in 1 of 7 mice in the I.V. administration group, whereas successful engraftment was obtained in 3 of the 3 recipient mice in the P.V. administration group. This data clearly demonstrates that unexpectedly superior results are obtained using TBI, and a sub-lethal irradiation dose of at least 6.5 Gy in combination with P.V. administration (see the attached Declaration).

Applicants respectfully submit that the Examiner has failed to address Applicants' arguments as to why there was no motivation to combine the teachings of the cited references

and has failed to address the data provided in the specification which demonstrates unexpectedly superior results (see pages 5-6 of the Amendment filed May 1, 2001).

As to Zhang et al, this reference merely discloses the results comparing I.V. with P.V. in a system wherein irradiation is not conducted. As the Examiner has previously admitted, the results obtain in this system can not be transferred to a system wherein irradiation is conducted, as in the present invention.

More specifically, it is apparent to ones skilled in the art that the results obtained by the non-TBI system (TBI dose of 0 Gy) disclosed in Zhang et al can not be applied to a TBI system. In other words, a skilled artisan would not foresee the effects achieved by TBI + P.V. from the teachings of Ildstad et al in view of Zhang et al. This is clear from the Examiner's assertion that Hayashi et al and Takao et al (of record) can not be used to infer the results of Ildstad et al. That is, it is the Examiner's position that even in systems with irradiation, if the radiation doses are different, the results can not be inferred. Thus, in view of the Examiner's assertion, there is no way that a person skilled in the art could predict that similar effects can be attained when the techniques used in a non-irradiation system (Zhang et al) are employed in a system with irradiation (Ildstad et al).

In summary, the claims set forth an engraftment rate and a one-day administration protocol which are not taught or suggested in Zhang et al and Ildstadt et al. Thus, even if Ildstadt et al and Zhang et al were combined with Slavin et al, the present invention would not be achieved. In any event, while Applicants do not believe that the Examiner has established *prima facie* case of obviousness, the evidence

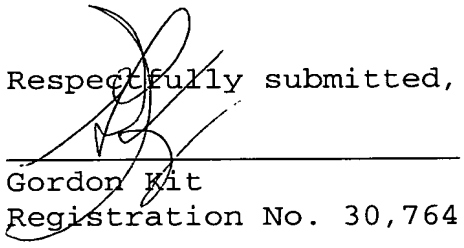
of record is clearly sufficient to rebut any such *prima facie* case of obviousness.

Accordingly, Applicants respectfully submit that the Examiner's rejection is improper and request withdrawal thereof.

In view of the arguments set forth above, reexamination, reconsideration and allowance are respectfully requested.

The Examiner is invited to contact the undersigned at the telephone number listed below on any questions that might arise.

Respectfully submitted,



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TREATMENT OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS BY TRANSPLANTATION OF ISLET CELLS PLUS BONE MARROW CELLS VIA PORTAL VEIN IN RATS[1]

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Background. **We have established a new method for the transplantation of allogeneic pancreatic islets (PIs) using sublethal irradiation (9 Gy) plus simultaneous transplantation of PIs and bone marrow cells (BMCs) via the portal vein (PV) followed by intravenous (i.v.) injection of donor BMCs (9 Gy + PV + i.v.).**

Methods. **Approximately 600 PIs of Brown Norway (BN: RT1A^n, RT1B^n) rats were transplanted into the liver of streptozotocin-induced diabetic Fischer 344 (F344: RT1A^l, RT1B^l) rats via the PV. BMCs (3×10^8) of BN rats were injected via the PV or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given 5 days after the PI transplantation.**

Results. **All the recipients (10 of 10) in the 9 Gy + PV + i.v. group showed normoglycemia for more than 1 year, whereas PIs were rejected within 30 days after transplantation in the group of 9 Gy + i.v. + i.v.**

Conclusions. **These results suggest that simultaneous transplantation of PIs and BMCs via the PV is effective in inducing persistent tolerance.**

Insulin-dependent diabetes mellitus (IDDM) is mainly juvenile-onset, nonobese, and ketosis-prone diabetes. In IDDM, insulin-producing beta cells of the pancreatic islets (PIs) are progressively destroyed, insulin production is reduced, and the plasma insulin level becomes extremely low (*1*). Exogenous insulin injection has prolonged the survival of diabetic patients and has prevented systemic complications (*2*). However, it is difficult to tightly control glucose homeostasis using insulin injections. The transplantation of isolated PIs is a physiologic approach to the replacement of pancreatic endocrine functions, and is more easily performed than whole pancreas transplantation. It has been recognized that transplanted isolated PIs are functional as a regulator of blood glucose (*3*, *4*). Transplanted PI grafts with portal venous drainage are more efficient in normalizing glucose metabolism than systemically draining PI grafts (*5–8*). Although improved immunosuppressive agents and techniques for the isolation of large numbers of functional PIs have made PI transplantation a possible therapeutic approach for diabetic patients, rejection remains a major limitation preventing widespread clinical application.

It is well known that successful transplantation of allogeneic PIs is difficult because of their strong antigenicity (*9*). There are many problems in the allogeneic transplantation of PIs, a major one being the development of acute and chronic rejection despite continuous usage of immunosuppressants. In addition, side effects of continuous immunosuppression are reported: these include the development of malignancies, opportunistic infections, and organ toxicity. The first association between bone marrow chimerism and tolerance was reported in 1953 (*10*). The donor-specific tolerance induced by bone marrow chimerism was found to eliminate the problem of allograft rejection (*11–15*).

It is also well known that the portal venous administration of alloantigens can induce tolerance. Callery et al. (*16*) and Genden et al. (*17*) have reported that the administration of donor cells via the portal vein (PV) promotes peripheral donor-specific hyporesponsiveness and prevents allografts of organs and tissues.

We have previously found that the administration of allogeneic cells via the PV induces donor-specific tolerance across MHC barriers (*18*), and that donor hemopoietic stem cells (HSCs), which are trapped in the liver after PV injection, induce anergy to host CD8$^+$ T cells owing to the absence of costimulatory signals (*19*). We have also found that the injection of HSCs via the PV plus short-term administration of an immunosuppressant (cyclosporine or FK-506) can induce persistent tolerance in the skin allografts in mice (*20*) and pigs (*21*).

In the present study, we show a strategy to allow the long-term acceptance of allogeneic PIs with normal functions without recourse to the use of immunosuppressants: 9 Gy irradiation and intrahepatic transplantation of PIs plus PV injection of whole donor bone marrow cells (BMCs), followed by i.v. injection of whole donor BMCs 5 days after PI transplantation.

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MATERIALS AND METHODS

Animals

Eight- to 10-wk-old male Brown Norway (BN, RT1A^n, RT1B^n) and 7- to 9-wk-old female Fischer 344 (F344, RT1A^l, RT1B^l) rats were purchased from Clea Japan Inc. (Tokyo, Japan) as donors and recipients, respectively, and maintained in our conventional animal facilities.

Pharmacologic Induction of Diabetes

Diabetes mellitus was induced in normal F344 rats by a single i.p. injection of 70 mg/kg of streptozotocin (Nacalai Tesque, Osaka, Japan), which is directly toxic to beta cells and induces an outbreak of clinical diabetes within 3 days (*22*). Diabetic F344 rats that showed more than 400 mg/dl for at least 3 consecutive days in nonfasting plasma glucose were used as recipients.

Transplantation

PIs were isolated by the collagenase technique as previously described (*23*). Diabetic F344 rats were irradiated (8, 8.5, or 9 Gy) with a single dose from a ^{137}Cs source 1 day before transplantation. Under pentobarbital (Essex Animal Health Friesoythe, Friesoythe, Germany) anesthesia, the peritoneal cavity of the recipient was accessed through a midline incision. The 600 PIs from BN rats were transplanted into the liver via the PV using a 26-gauge needle. After infusion, the syringe was rinsed several times by repeated aspiration and reinfusion of PV blood. BMCs were collected from the femurs and tibias of BN rats, and 3×10^8 whole BMCs were injected intraportally (PV) or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given via the lateral tail vein 5 days after the PI transplantation. Several experimental groups were set up: (1) 9 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+PV+i.v., n=10), (2) 9 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+i.v.+i.v., n=7), (3) 9 Gy irradiation plus PV injection of PIs and BMCs (9 Gy+PV; n=10), (4) 8.5 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+PV+i.v., n=9), (5) 8.5 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+i.v.+i.v., n=7), (6) 8.5 Gy irradiation plus PV injection of PIs and BMCs (8.5 Gy+PV; n=7), and (7) 8 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs (8 Gy+PV+i.v., n=8). The graft-accepting recipients showed no morbidity (diarrhea, cachexia, or alopecia, etc.) after transplantation.

Normal F344 rats did not die after 9 Gy irradiation because of the recovery of their own BMCs even when no BMCs were injected (data not shown). Therefore, we conclude that 9 Gy is a sublethal irradiation dose for rats.

Criteria for Rejection

Graft rejection was considered to have occurred when posttransplantation nonfasting plasma glucose levels exceeded 300 mg/dl for two consecutive measurements.

Flow Cytometric Analyses

MHC class I (RT1A^l or RT1A^n) surface markers were examined for the peripheral blood mononuclear cells (PBMCs) of recipients on the days after transplantation indicated in Figure 3 by a FACScan (Becton Dickinson, Mountain View, CA). Briefly, peripheral blood was layered over lymphocyte separation medium (Lympholyte-Mammal, Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) and centrifuged at 18°C (5000 rpm for 30 min). The lymphocyte layer was aspirated from the serum-lymphocyte separation medium interface and washed. Cells were stained with fluorescein isothiocyanate-labeled anti-RT1A^l monoclonal antibodies (mAbs) against recipient MHC class I (RT1A^l, PharMingen, San Diego, CA), and mAbs against donor MHC class I (RT1A^n, Serotec, Oxford, England), followed by staining with phycoerythrin-labeled anti-mouse IgG Abs (Serotec).

Glucose Tolerance Tests

Five months after the PI transplantation, glucose tolerance tests (GTTs) were performed. Food was removed for 16 hr before the GTTs were started. The rats in each experimental group were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained 15, 30, 60, and 120 min after the glucose injection. Plasma glucose levels were analyzed by a glucose oxidase method.

Serum Insulin Measurements

Food was removed for 16 hr before the serum insulin levels were measured. Rats were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained at 30 min after glucose injection and measured for serum insulin levels using an ELISA method (*23*).

Histologic Findings

The livers of the recipient rats were removed and fixed in 10% buffered formalin, processed for light microscopy, and stained with hematoxylin and eosin (H-E) and with anti-insulin antibody to identify the grafted PIs.

Mixed Lymphocyte Reaction

For mixed lymphocyte reaction (MLR), splenic T cells were obtained by passing spleen cells through a nylon-wool fiber (Wako Pure Chemical, Osaka, Japan) column after incubation for 60 min at 37°C; 1 g of the nylon-wool fiber was prepared for 3×10^7 splenocytes to use as responders. Lymphocytes (3×10^6) were cocultured with 20 Gy-irradiated whole spleen cells (3×10^6: stimulator cells) in a total of 200 μl of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 50 μM of 2-mercaptoethanol (2-ME; Wako). The cultures were incubated at 37°C for 5 days in a humidified 5% CO_2 environment in 96-well flat-bottom microwell trays (Corning Glass Works, Corning, NY), and pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culture period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.

Assay for Generation of Cytotoxic T Lymphocytes

Cytotoxic T-lymphocyte (CTL) assays were evaluated by measuring the release of lactate dehydrogenase (LDH) from target cells using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kits (Promega, Madison, WI), following the manufacturer's instructions. Briefly, splenocytes from F344 (RT1A^l), BN (RT1A^n), or ACI (August Copenhagen Irish; RT1A^a) rats were cultured with 2.5 mg/ml concanavalin A (Con A, Sigma, St. Louis, MO) for 3 days to prepare the Con A blastocytes as target cells. After washing, 3×10^5/well of Con A blastocytes were incubated for another 12 hr with or without 1×10^6/well of splenocytes from posttransplantation F344 or BN rats.

Statistical Analyses

Statistical analyses were performed using a two-tailed Student's *t*-test, except for graft survival rates. Statistical analyses of graft survival rates were performed using a log rank test.

RESULTS

Graft Survival

Because we have found that the injections of BMCs via the PV plus i.v. are effective in inducing persistent tolerance not only in chimeric-resistant MRL/lpr mice (*24*) but also in skin allografts of mice (*20, 25*) and pigs (*21*), we have attempted to examine the effect of PV injection of BMCs on tolerance induction using the PI transplantation system. To detect the

diabetic condition, nonfasting blood glucose levels were monitored every other day in F344 rats in which diabetes mellitus had been induced by streptozotocin. Plasma glucose levels in all recipient rats returned to normal within 24 hr after the PI transplantation (Fig. 1A). We monitored the graft survival by measuring nonfasting blood glucose levels. In the 8 Gy+PV+i.v., 8.5 Gy+i.v.+i.v., and 9 Gy+i.v.+i.v. groups, all grafts were rejected within 30 days. The 9 Gy+PV group showed a 70% graft survival rate 320 days after transplantation, whereas the 9 Gy+PV+i.v. group showed a 100% graft survived rate more than 1 year after the PI transplantation. All rats (10 of 10) in the 9 Gy+PV+i.v. group showed normoglycemia for more than 1 year. The graft survival rate in the 8.5 Gy+PV+i.v. group was 44% 150 days after transplantation (Fig. 1). In the 8.5 Gy+PV group, only one in seven cases accepted the PIs until day 185. These results suggest that simultaneous PV injection of BMCs induces tolerance to allogeneic PIs more easily than the i.v. injection of BMCs, and that the additional i.v. injection of BMCs maintains the tolerance induced by the PV injection of BMCs.

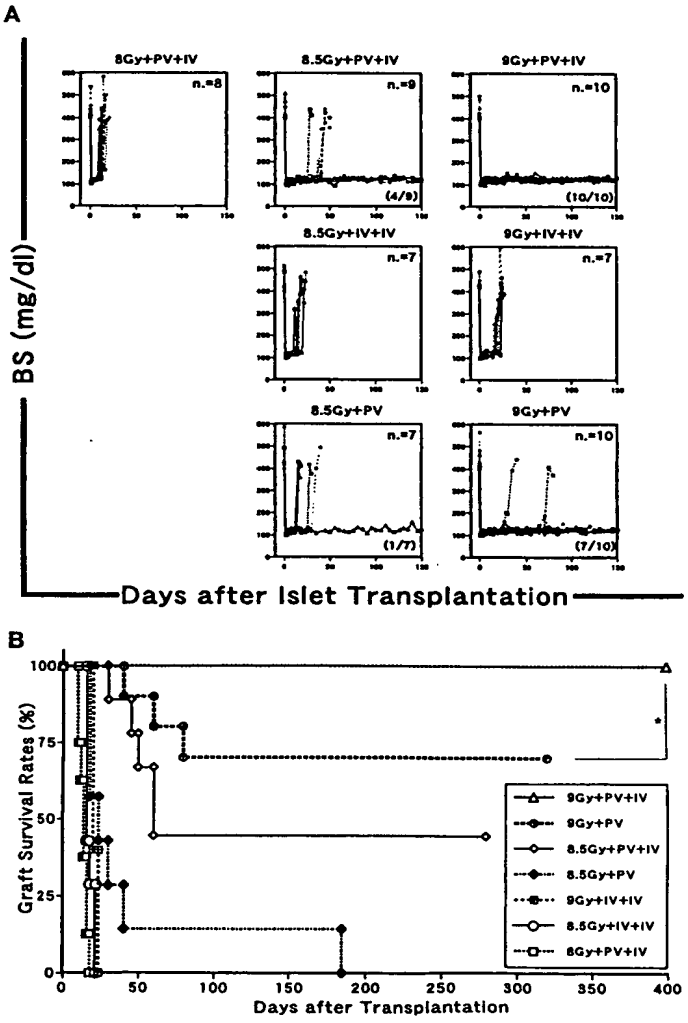

FIGURE 1. Graft survival of transplanted PIs. After irradiation, the PIs of BN rats were transplanted into F344 rats with BMCs of BN rats via the PV or i.v. Additional i.v. injection of BMCs from BN rats was performed in some groups, as described in *Materials and Methods*. Graft survival terms (A) and graft survival rates (B) in each experimental group are shown (*$P<0.01$). BS, blood sugar.

Histologic Findings

The livers of recipient rats were stained with H-E and with anti-insulin antibody to identify the grafted PIs. Grafted PIs were found in the 9 Gy+PV+i.v. group (60 days after transplantation; Fig. 2A), whereas disrupted islets, into which lymphocytes and macrophages had infiltrated, were seen in the livers of the 9 Gy+i.v.+i.v. group 30 days after transplantation (Fig. 2B). In the livers of the 9 Gy+PV+i.v. group, the grafted PIs were found even 200 days after the transplantation (Fig. 2C), although they were scattered throughout the liver in contrast with their distribution at 60 days after transplantation. The PIs grafted via the PV formed clusters in the liver for a short time, but they later became scattered throughout the liver. This seems to be related to the fact that the hepatocytes can proliferate, while the PI cells cannot.

Insulin was positively stained in the grafted PIs (Fig. 2D). In contrast, infiltrating mononuclear cells and fibrosis were seen in the livers of the 9 Gy+i.v.+i.v. group, and no insulin-positive PIs were found in the livers (data not shown). These results suggest that functional PIs exist in the livers of normoglycemic recipient rats, and that the grafted PIs are destroyed by immunologic mechanisms in the livers of hyperglycemic recipient rats.

Flow Cytometric Analyses of PBMCs

Chimerism was evaluated by flow cytometric analyses 90 days after transplantation using recipient PBMCs and anti-class I rat mAbs. The PBMCs in all graft-accepting rats showed the donor phenotype, although those of one graft-accepting rat in the 8.5 Gy+PV group partially showed the recipient phenotype, the level being 18.4% on day 90. Moreover, the transplanted PIs of this rat were rejected on day 185 when PBMCs showed only the recipient phenotype. The PBMCs in all the graft-rejecting rats showed the recipient phenotype (Fig. 3). However, all the PI-transplanted rats treated with 9 Gy+PV+i.v. survived more than 1 year, and their PBMCs were donor-type cells (>98%; Fig. 3). These results suggest that the existence of donor bone marrow-derived cells is crucial for successful transplantation of the PIs.

Intravenous Glucose Tolerance Tests and Serum Insulin Levels. As nonfasting blood glucose levels do not provide an accurate assessment of true regulation of glucose homeostasis in the PI graft-accepting recipients, i.v. glucose tolerance tests (IVGTTs) were performed and the serum insulin levels measured to evaluate the functions of the grafted PIs 150 days after transplantation. We compared the IVGTTs between the rats treated with 9 Gy+PV+i.v. and untreated control F344 rats (Fig. 4A). Fasting blood glucose levels were not significantly different between the graft-accepting rats (n=6) and untreated normal control rats (n=6). At 15 and 30 min after glucose administration, the PI-accepting rats showed a slightly higher blood glucose level than untreated normal control rats, although there was no significant difference. At 60 and 120 min after glucose administration, the blood glucose levels in the PI-accepting rats and untreated normal control rats returned to the levels before glucose administration and showed similar values.

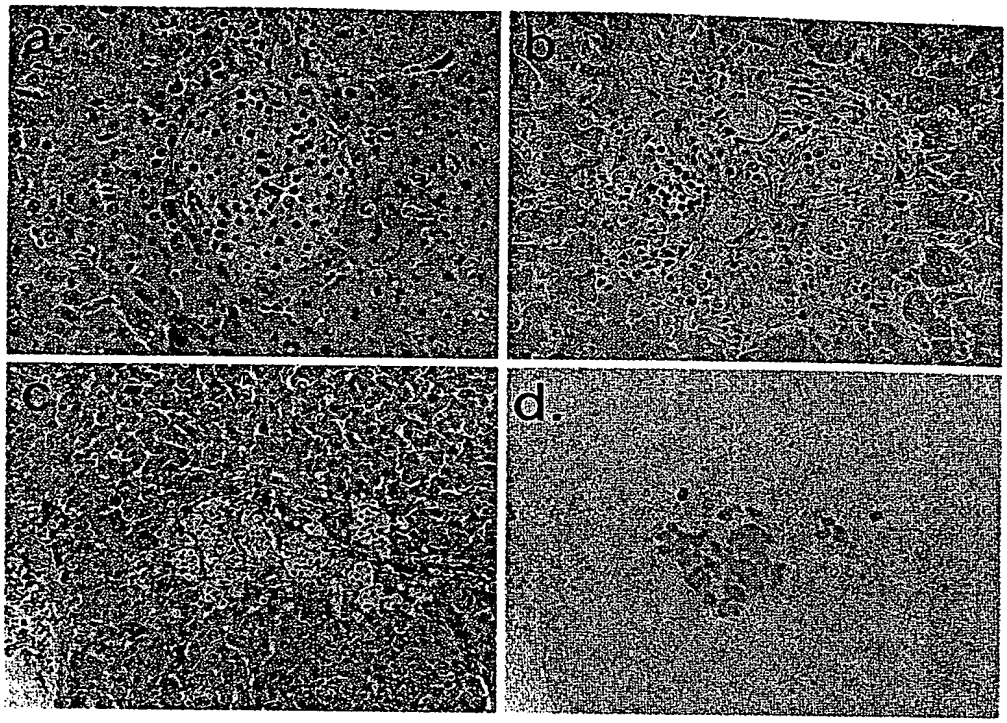

FIGURE 2. Histologic findings of transplanted PIs. PI-transplanted rats were killed 60 days after transplantation, followed by the histologic study of the livers, into which allogeneic PIs had been transplanted via the PV. Histologic examination was performed as described in *Materials and Methods*. The liver of a PI-transplanted rat with normoglycemia in nonfasting blood (a, H-E staining) and the liver of a PI-transplanted rat with hyperglycemia (b, H-E staining) are shown. PI-transplanted rats with normoglycemia were killed also 180 days after transplantation, and representative data on H-E staining (c) or immunohistologic staining for insulin (d) are shown.

We next measured the serum insulin levels in the groups of untreated normal control F344 rats (n=7), diabetes mellitus (DM)-induced nongrafting rats (n=7), PI-rejecting rats (n=7), and PI-accepting rats (n=12; Fig. 4B). The insulin levels in the group of DM-induced and PI-rejecting rats were significantly lower than those in the group of untreated normal rats. The insulin levels in PI-accepting rats were significantly higher than those in the PI-rejecting rats and were slightly lower than those in untreated normal rats, although there was no significant difference. These results suggest that the insulin levels of the DM rats do not recover owing to the destruction of beta cells, and that glucose homeostasis is maintained by the grafted PIs. The insulin levels in the rats treated with 9 Gy irradiation plus PV injection of only BMCs (without grafting PIs) followed by i.v. injection of BMCs after 5 days were similar to those of DM-induced rats (data not shown).

Mixed Lymphocyte Reaction

The splenic T cells of recipient rats that had received the PIs and BMCs from the donors were examined for their reactivity to the recipient, donor, and third-party alloanti-

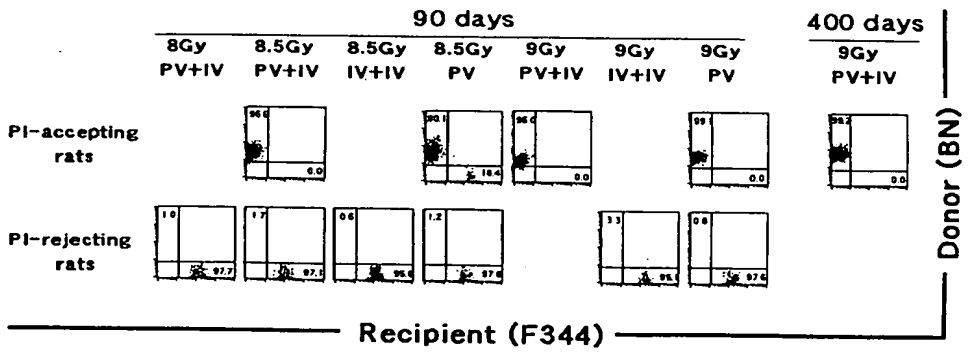

FIGURE 3. Fluorescence-activated cell sorter analyses using PBMCs of posttransplanted rats. The phenotypes of MHC class I in the PBMCs obtained from the PI-transplanted rats 90 days after various treatments (as indicated in this figure) or 400 days after the treatment with PV+i.v. were analyzed using a FACScan. Representative data in each experimental group are shown.

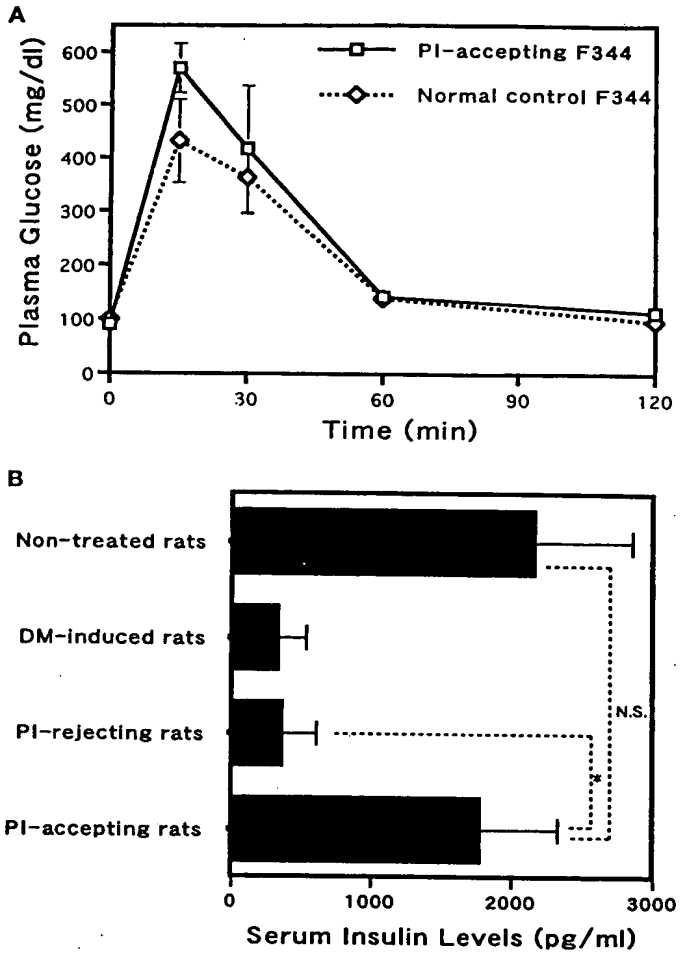

FIGURE 4. (A) IVGTTs of PI-transplanted rats in the 9 Gy+PV+i.v. group with normoglycemia in nonfasting blood were performed, and compared with those of untreated normal control rats. (B) Normal serum insulin levels of PI-accepting rats. Serum insulin levels were measured 150 days after PI transplantation, as described in *Materials and Methods* (*$P<0.005$, N.S., not significant).

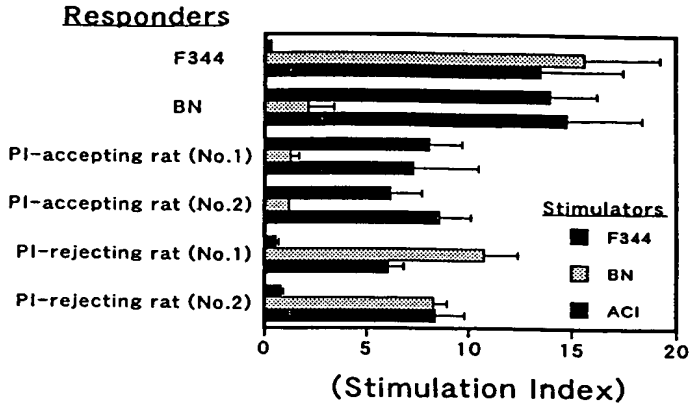

FIGURE 5. MLR in T cells from posttransplanted rats. MLR assays were performed as described in *Materials and Methods*. Splenocytes (3×10^7) as stimulators from untreated F344, BN, and ACI rats were cultured for 5 days with or without T cells (3×10^6) of posttransplanted F344, BN, or ACI rats. They were pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culturing period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.

gens using MLR assays 150 days after transplantation (Fig. 5). Untreated normal control F344 and BN rats showed normal proliferative responses to stimulator cells. The T cells of the graft-rejecting rats showed no response to the splenic cells of recipient F344 rats, although they did show responses to both donor and third-party MHC determinants. In contrast, the T cells of graft-accepting recipients, which were tolerant to donor alloantigens, showed similar proliferative responses to recipient stimulator cells as well as MHC-disparate third-party rat stimulator cells. Thus, the T cells of the rats with successful transplants responded to not only third-party cells but also host-type cells, although the graft-accepting recipients did not show any symptoms of graft-versus-host reaction (GvHR) for more than 1 year.

CTL Assays

Inasmuch as the graft-accepting rats showed the proliferative responses to host splenocytes in MLR assays but showed no symptoms of GvHR, we next performed the assays for the generation of CTLs to examine whether transplanted HSCs show cytotoxic effects on recipient cells in vitro. As shown in Figure 6, the splenocytes of F344 rats showed cytotoxic effects on both BN rat and third-party ACI rat cells, and the splenocytes of the donor BN rats showed cytotoxic effects on the recipient F344 rat cells. However, the graft-accepting rats showed no cytotoxic effects on not only donor rat (BN) cells but also recipient rat (F344) cells, although they showed a significant response to ACI (a third-party) rat cells. These results suggest that the transplanted donor HSCs do not injure the host cells even in vitro, although the T cells of the graft-accepting rats showed the proliferative responses to host cells in MLR assays.

DISCUSSION

Successful transplantation of tissues, cells, and organs between fully MHC-mismatched donor and recipient combinations has been dependent on the use of immunosuppressive agents to control acute and chronic rejection. Immunosuppressants have some toxic effects on various cells, which results in significant morbidity and mortality. It has been recognized that the induction of donor-specific tolerance by bone marrow chimerism can eliminate the problem of allograft rejection (*11–15*). Although bone marrow chimerism can successfully prevent even chronic graft rejection, the clinical application of the lethal conditioning approach to induce tolerance would be limited by the excessive toxicity associated with lethal conditioning.

The administration of insulin for the treatment of IDDM usually controls blood glucose and prevents lethal diabetic ketoacidosis. However, even frequent insulin treatments are not enough to prevent the high morbidity and mortality associated with IDDM (*26*). It has been reported that the transplantation of whole pancreas can maintain normal blood glucose levels and effectively control IDDM (*27*). The transplantation of the endocrine tissue alone (isolated PIs) is a

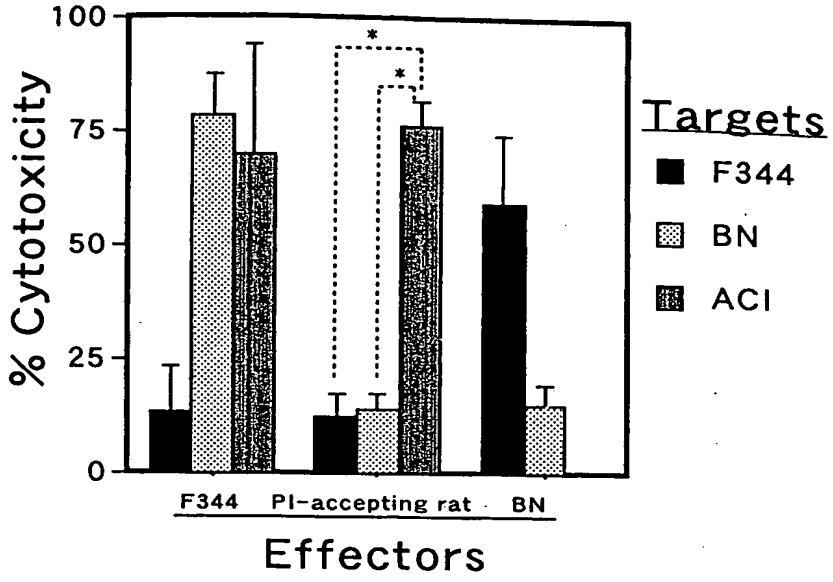

FIGURE 6. CTL assays. Con A blastocytes (3×10^5), which were prepared from splenocytes (1×10^6) of F344, BN, and ACI rats, were cultured for 12 hr with or without splenocytes from posttransplanted F344 or ACI rats, followed by measuring the levels of LDH in the wells using CytoTox 96 (*$P<0.05$).

more technically simple approach than whole pancreatic transplantation. It has also been reported that the survival term of the grafts in the transplantation of PIs is short owing to their high antigenicity and their high sensitivity to graft rejection (*28*).

It has been reported that systemically draining grafts (such as PI grafts under the renal capsule) directly secrete insulin into the systemic circulation and induce hyperinsulinemia (*29*). Therefore, the transplantation of PIs via the PV (PV drainage) is a more physiologic approach than systemic drainage (*5, 8*), and intrahepatic PI transplantation is easily performed. Clinically, PV injection is easily carried out under echographically guided portal puncture with local anesthesia. We have previously found that the PV administration of allogeneic cells can induce donor-specific tolerance across MHC barriers (*18*), and that the injection of BMCs via the PV followed by transient usage of an immunosuppressant without irradiation can induce persistent tolerance in skin allografts (*20, 21*). In the present study, we have found a strategy for long-term acceptance of PIs with normal functions without recourse to immunosuppressants: a sublethal irradiation dose (9 Gy) plus simultaneous injection of BMCs and PIs via the PV followed by i.v. injection of BMCs. The 9 Gy+PV+i.v. group showed normal glucose levels and a 100% survival rate, and did not develop IDDM by 400 days after transplantation (Fig. 1), whereas the 9 Gy+i.v.+i.v. group showed high glucose levels owing to the rejection of the PIs (Fig. 1A). The PBMCs in all but one graft-accepting rat showed the donor phenotype. The exception was in the 8.5 Gy+PV group; 18.4% recipient phenotype was detected in the PBMCs in this rat at 90 days after transplantation, indicating that mixed chimerism existed at that time. The PI graft of this rat was rejected 185 days after transplantation, at which time no donor cells were detected in the PBMCs. The mechanism underlying the loss of mixed chimerism has not been elucidated, but the loss may reflect the presence of MHC restriction (preference) between HSCs and stromal cells, as we previously described (*30, 31*). Therefore, the complete replacement of hemopoietic cells by donor cells (instead of mixed chimeras) is crucial to the induction of persistent tolerance, as we have previously shown in lethally irradiated mice (*1, 11–13, 24*). We have previously used T-cell–depleted BMCs for allogeneic bone marrow transplantation (BMT). However, we have recently used whole BMCs, which contain a small number of T cells (<1%). The T cells present in the bone marrow were found not to induce GvHR, but to facilitate bone marrow engraftment (prevent HvGR) (*32, 33*), even when sublethal irradiation doses were used for allogeneic BMT (*34*) and organ transplantation (*25*). It should be noted that sublethal irradiation (7 Gy for mice (*25*) and 9 Gy for rats in the present study) induces fully allogeneic chimerism (>98%) for more than 1 year without evidence of either GvHR or HvGR.

It is thought that IDDM is an organ-specific autoimmune disease, which is characterized by the destruction of insulin-producing beta cells by autoimmune mechanisms (*1*). The nonobese diabetic (NOD) mouse is a well-known animal model for IDDM. We have previously shown that allogeneic BMT can prevent and treat insulitis (*1*), and that allogeneic BMT plus fetal pancreas grafts can treat overt diabetes in NOD mice (*12*). However, we have found that in (BALB/c+NOD→NOD) chimeric mice, NOD hemopoietic cells become dominant, which results in the development of IDDM (*35*), because the abnormal HSCs of autoimmune-prone mice are more resilient than normal HSCs, as we previously described (*36*). These findings suggest that allogeneic BMT instead of mixed allogeneic BMT should be carried out in conjunction with organ transplantation.

Hemopoietic cells from PI-accepting rats showed the proliferative response to host cells in MLR assays in vitro. However, they showed no cytotoxic effects on host cells not only in vivo (no GVHR) but also in CTL assays in vitro. This split tolerance, as previously described by Sprent et al. (*37*), is interesting for analyzing the mechanisms underlying tolerance induction. It is conceivable that some suppressor mechanisms that inhibit the functions of cytotoxic effector cells

against the host are involved. We are now in the process of examining regulatory and suppressor cells in our system.

In conclusion, we have demonstrated that the combination of PV plus i.v. injections of BMCs is effective in inducing donor-specific tolerance across MHC barriers, even when the irradiation dose is reduced to 9 Gy (sublethal dose). This strategy (9 Gy+PV+i.v.) leads to long-term acceptance of PIs with normal functions, enabling the treatment of diabetes mellitus without recourse to immunosuppressants.

Acknowledgments. The authors thank Y. Tokuyama, M. Shinkawa, and S. Miura for their expert technical assistance, and Hilary Eastwick-Field and K. Ando for their help in the preparation of the manuscript.

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susumu IKEHARA et al	Confirmation No.: **2802**
Appln. No.: **09/380,579**	Group Art Unit: **1644**
Filed: **September 7, 1999**	Examiner: **Belyavskyi, M.**

For: **METHOD OF INDUCING IMMUNOTOLERANCE IN AN ORGAN TRANSPLANTATION RECIPIENT**

DECLARATION UNDER 37 C.F.R.§ 1.132 OF SUSUMU IKEHARA

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Susumu Ikehara do hereby declare and state:

I received an M.D. from Kyoto University, and a Ph. D. from Kyoto University.

From 1976-1982, I was a Lecturer in Department of Pathology, Kyoto University.

From 1978-1981, I was a Visiting Investigator at the Memorial Sloan-Kettering Cancer Center in New York.

From 1982-1984, I was an Associate Professor in the Department of Pathology, Kansai Medical University.

Since 1985, I have been a Professor in the Department of Pathology, Kansai Medical University.

In 1992, I was a Visiting Professor at Norman Bethune University of Medical Sciences.

Since 1998, I have been an *Emeritus* Professor at Norman Bethune University of Medical Sciences.

From 1998-2000, I was the Director of Transplantation Center, Kansai Medical University.

DECLARATION UNDER 37 C.F.R.
§ 1.132 OF SUSUMU IKEHARA
U.S. Appln. No. 09/380,579

From 2001-2002, I was the Director of Regeneration Research Center for Intractable Diseases, Kansai Medical University.

Since 2003, I have been the Director of Center for Cancer Therapy, Kansai Medical University.

I am a co-inventor of the invention disclosed and claimed in the above-identified application.

I am familiar with the Office Action dated October 20, 2003, and also the Advisory Action dated March 4, 2004, in the above-identified application, wherein the Examiner reject Claims 9-10 under 35 C.S.C.§ 103 as being obvious over Slavin et al in view of Ildstad et al and Zhang et al.

In order to demonstrate the unobviousness of the present invention over the cited references, I further hereby declare and state:

Ildstad et al relates to a technique using mixed chimerism, and is fundamentally different from the present invention, which uses fully allogenic chimerism.

Ildstad et al discloses that "allogenic engraftment was reliably achieved in 100%" by employing 7.0 Gy + intravenous injection (I.V.) (see Figure 1 and column 17, lines 15-16 in Ildstad et al). However, this merely indicates that the 100% of the animals conditioned by 7.0 Gy + I.V. exhibited mixed chimerism. This is clear form the expression "% recipients with chimerism" for the vertical axis in Figure 1, and the description at column 17, lines 9-15, thereof.

This is entirely different from the effects of the present invention, i.e., "transplanting an organ into said recipient, to

thereby achieve an engraftment rate of 100%".

Contrary to the Examiner's contention in the Final Office Action dated July 29, 2002, Figure 7 of Ildstad et al does not show a 100% acceptance of skin grafts after 30 days; the only skin grafts showing no rejection after 30 days are (unsurprisingly) those of the recipients themselves. On the other hand, donor-specific grafts were already approximately 10% rejected after 20 days.

This is to be expected since, as disclosed in Ikebukuro et al, *Transplantation*, 73(4):512-518 (2002) and Hayashi et al, *Stem Cells*, 18:273-280 (2000), when a mixed chimerism technique is employed, donor-derived BMCs gradually decrease, leading eventually to organ rejection.

The Examiner relies on his assertion that Ildstad et al teaches 100% skin graft acceptance after 30 days to support his position that 100% engraftment of BMCs in Ildstad et al is equivalent to a 100% organ engraftment rate in the present invention, and therefore contends that it is proper to combine Ildstad et al with Slavin et al.

However, because a skilled person in the art would note, as shown in Figure 7 in Ildstad et al, that a 100% engraftment rate for BMCs does not lead to 100% organ engraftment, the skilled person would not be motivated to combine Ildstad et al with Slavin et al.

As discussed above, it can not be expected to achieve the effect essential to the present invention, i.e., the transplanted organ survives for a long time and fulfils its function, by employing

the technique disclosed in Ildstad et al. Furthermore, there is no description or data in Ildstad et al from which a skilled artisan could predict or expect such an effect.

Takao et al, *Immunobiology*, 194:376-389 (1995) discloses that the technique for forming mixed chimeras by bone marrow transplantation taught by Ildstad et al does not interfere with the redevelopment of diabetes when employed with insulin-dependent diabetes (IDDM) mice (NOD), and that hematolymphoid cells of NOD mice must be completely destroyed by irradiation to prevent redevelopment of diabetes (see Abstract).

Ikebukuro et al, *supra*, teaches in relation to employment of whole pancreas transplantation, which seems to be effective for treating diabetes such as disclosed in Takao et al, *supra*, for that all of the rats which received sublethal irradiation (9 Gy) plus simultaneous transplantation of PIs and BMCs via the portal vein (P.V.) followed by I.V. of donor EMCs, showed normoglycemia for more than one year. However, in the rats receiving 9 Gy + I.V. + I.V., PIs were rejected within 30 days after transplantation (see Abstract).

From this description, it is clear that rejection of such a transplant is unavoidable using the technique taught by Ildstad et al.

In the experiment in Ikebukuro et al, *supra*, rats were used as the test animal and 9 Gy of total body irradiation (TBI) was the sublethal irradiation dose for the rats (although 7 Gy is the sublethal irradiation dose in mice). The difference between the irradiation doses is not the essential difference between the

teachings of Ildstad et al and Ikebukuro et al, *supra*. Both are the same in employing a sublethal irradiation dose.

Ikebukuro et al, *supra*, discloses that, in the technique using mixed chimerism as taught by Ildstad et al, mixed chimerism is not permanent, i.e., the donor-derived PMBCs gradually decrease and eventually disappear, and accordingly, the transplanted PIs do not survive in the long term after transplantation. Rather, they are rejected after a while (page 517, left column, lines 11-6 from the bottom).

Therefore, it is clear that the technique of Ildstad et al is not usable for an organ transplantation.

Hayashi et al, *supra*, also discloses that the transplanted donor's cells gradually decrease in mice when the technique of mix chimerism, as taught by Ildstad et al is employed (in particular, see Figure 1 on page 276). As is also clear from the description of Hayashi et al, *supra*, the present invention exhibits remarkable effects, i.e., successful maintenance of a graft organ, that can not be predicted by the teaching of Ildstad et al.

As shown in Figure 2 of the present specification, the recipient mice given an irrediation dose of 7 Gy in the P.V. administration group showed an engraftment rate of 100% after transplantation. This is in contrast with the recipient mice exposed to an irradiation dose of 6.0 Gy in which the skin graft was invariably rejected within 3 weeks after transplantation. In recipient mice given an irradiation dose of 6.5 Gy, the skin graft was rejected in 1 of 7 mice in the I.V. administration group within 3 weeks after

transplantation, whereas successful engraftment was obtained in 3 of the 3 recipient mice in the P.V. administration group for 13 weeks after transplantion. This data clearly demonstrates that unexpectedly superior results are obtained using TBI in a sublethal irradiation dose of at least 6.5 Gy in combination with P.V. administration as recited in Claims 9-10.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: June 4, 2004 Name: Susumu Ikehara
Dr. Susumu Ikehara

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TRANSPLANTATION

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TREATMENT OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS BY TRANSPLANTATION OF ISLET CELLS PLUS BONE MARROW CELLS VIA PORTAL VEIN IN RATS[1]

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Background. **We have established a new method for the transplantation of allogeneic pancreatic islets (PIs) using sublethal irradiation (9 Gy) plus simultaneous transplantation of PIs and bone marrow cells (BMCs) via the portal vein (PV) followed by intravenous (i.v.) injection of donor BMCs (9 Gy + PV + i.v.).**

Methods. **Approximately 600 PIs of Brown Norway (BN: RT1A^n, RT1B^n) rats were transplanted into the liver of streptozotocin-induced diabetic Fischer 344 (F344: RT1A^l, RT1B^l) rats via the PV. BMCs (3×10^8) of BN rats were injected via the PV or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given 5 days after the PI transplantation.**

Results. **All the recipients (10 of 10) in the 9 Gy + PV + i.v. group showed normoglycemia for more than 1 year, whereas PIs were rejected within 30 days after transplantation in the group of 9 Gy + i.v. + i.v.**

Conclusions. **These results suggest that simultaneous transplantation of PIs and BMCs via the PV is effective in inducing persistent tolerance.**

Insulin-dependent diabetes mellitus (IDDM) is mainly juvenile-onset, nonobese, and ketosis-prone diabetes. In IDDM, insulin-producing beta cells of the pancreatic islets (PIs) are progressively destroyed, insulin production is reduced, and the plasma insulin level becomes extremely low (*1*). Exogenous insulin injection has prolonged the survival of diabetic patients and has prevented systemic complications (*2*). However, it is difficult to tightly control glucose homeostasis using insulin injections. The transplantation of isolated PIs is a physiologic approach to the replacement of pancreatic endocrine functions, and is more easily performed than whole pancreas transplantation. It has been recognized that transplanted isolated PIs are functional as a regulator of blood glucose (*3, 4*). Transplanted PI grafts with portal venous drainage are more efficient in normalizing glucose metabolism than systemically draining PI grafts (*5–8*). Although improved immunosuppressive agents and techniques for the isolation of large numbers of functional PIs have made PI transplantation a possible therapeutic approach for diabetic patients, rejection remains a major limitation preventing widespread clinical application.

It is well known that successful transplantation of allogeneic PIs is difficult because of their strong antigenicity (*9*). There are many problems in the allogeneic transplantation of PIs, a major one being the development of acute and chronic rejection despite continuous usage of immunosuppressants. In addition, side effects of continuous immunosuppression are reported: these include the development of malignancies, opportunistic infections, and organ toxicity. The first association between bone marrow chimerism and tolerance was reported in 1953 (*10*). The donor-specific tolerance induced by bone marrow chimerism was found to eliminate the problem of allograft rejection (*11–15*).

It is also well known that the portal venous administration of alloantigens can induce tolerance. Callery et al. (*16*) and Genden et al. (*17*) have reported that the administration of donor cells via the portal vein (PV) promotes peripheral donor-specific hyporesponsiveness and prevents allografts of organs and tissues.

We have previously found that the administration of allogeneic cells via the PV induces donor-specific tolerance across MHC barriers (*18*), and that donor hemopoietic stem cells (HSCs), which are trapped in the liver after PV injection, induce anergy to host CD8$^+$ T cells owing to the absence of costimulatory signals (*19*). We have also found that the injection of HSCs via the PV plus short-term administration of an immunosuppressant (cyclosporine or FK-506) can induce persistent tolerance in the skin allografts in mice (*20*) and pigs (*21*).

In the present study, we show a strategy to allow the long-term acceptance of allogeneic PIs with normal functions without recourse to the use of immunosuppressants: 9 Gy irradiation and intrahepatic transplantation of PIs plus PV injection of whole donor bone marrow cells (BMCs), followed by i.v. injection of whole donor BMCs 5 days after PI transplantation.

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MATERIALS AND METHODS

Animals

Eight- to 10-wk-old male Brown Norway (BN, RT1A^n, RT1B^n) and 7- to 9-wk-old female Fischer 344 (F344, RT1A^1, RT1B^1) rats were purchased from Clea Japan Inc. (Tokyo, Japan) as donors and recipients, respectively, and maintained in our conventional animal facilities.

Pharmacologic Induction of Diabetes

Diabetes mellitus was induced in normal F344 rats by a single i.p. injection of 70 mg/kg of streptozotocin (Nacalai Tesque, Osaka, Japan), which is directly toxic to beta cells and induces an outbreak of clinical diabetes within 3 days (*22*). Diabetic F344 rats that showed more than 400 mg/dl for at least 3 consecutive days in nonfasting plasma glucose were used as recipients.

Transplantation

PIs were isolated by the collagenase technique as previously described (*23*). Diabetic F344 rats were irradiated (8, 8.5, or 9 Gy) with a single dose from a ^{137}Cs source 1 day before transplantation. Under pentobarbital (Essex Animal Health Friesoythe, Friesoythe, Germany) anesthesia, the peritoneal cavity of the recipient was accessed through a midline incision. The 600 PIs from BN rats were transplanted into the liver via the PV using a 26-gauge needle. After infusion, the syringe was rinsed several times by repeated aspiration and reinfusion of PV blood. BMCs were collected from the femurs and tibias of BN rats, and 3×10^8 whole BMCs were injected intraportally (PV) or i.v. into the recipients simultaneously. In some groups, additional i.v. injections of BMCs from BN rats were given via the lateral tail vein 5 days after the PI transplantation. Several experimental groups were set up: (1) 9 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+PV+i.v., n=10), (2) 9 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (9 Gy+i.v.+i.v., n=7), (3) 9 Gy irradiation plus PV injection of PIs and BMCs (9 Gy+PV; n=10), (4) 8.5 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+PV+i.v., n=9), (5) 8.5 Gy irradiation plus PV injection of PIs and i.v. injection of BMCs followed by i.v. injection of BMCs after 5 days (8.5 Gy+i.v.+i.v., n=7), (6) 8.5 Gy irradiation plus PV injection of PIs and BMCs (8.5 Gy+PV; n=7), and (7) 8 Gy irradiation plus PV injection of PIs and BMCs followed by i.v. injection of BMCs (8 Gy+PV+i.v., n=8). The graft-accepting recipients showed no morbidity (diarrhea, cachexia, or alopecia, etc.) after transplantation.

Normal F344 rats did not die after 9 Gy irradiation because of the recovery of their own BMCs even when no BMCs were injected (data not shown). Therefore, we conclude that 9 Gy is a sublethal irradiation dose for rats.

Criteria for Rejection

Graft rejection was considered to have occurred when posttransplantation nonfasting plasma glucose levels exceeded 300 mg/dl for two consecutive measurements.

Flow Cytometric Analyses

MHC class I (RT1A^1 or RT1A^n) surface markers were examined for the peripheral blood mononuclear cells (PBMCs) of recipients on the days after transplantation indicated in Figure 3 by a FACScan (Becton Dickinson, Mountain View, CA). Briefly, peripheral blood was layered over lymphocyte separation medium (Lympholyte-Mammal, Cedarlane Laboratories Ltd, Hornby, Ontario, Canada) and centrifuged at 18°C (5000 rpm for 30 min). The lymphocyte layer was aspirated from the serum-lymphocyte separation medium interface and washed. Cells were stained with fluorescein isothiocyanate-labeled anti-RT1A^1 monoclonal antibodies (mAbs) against recipient MHC class I (RT1A^1, PharMingen, San Diego, CA), and mAbs against donor MHC class I (RT1A^n, Serotec, Oxford, England), followed by staining with phycoerythrin-labeled anti-mouse IgG Abs (Serotec).

Glucose Tolerance Tests

Five months after the PI transplantation, glucose tolerance tests (GTTs) were performed. Food was removed for 16 hr before the GTTs were started. The rats in each experimental group were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained 15, 30, 60, and 120 min after the glucose injection. Plasma glucose levels were analyzed by a glucose oxidase method.

Serum Insulin Measurements

Food was removed for 16 hr before the serum insulin levels were measured. Rats were injected i.v. with 2 mg of glucose per gram of body weight. Blood samples were obtained at 30 min after glucose injection and measured for serum insulin levels using an ELISA method (*23*).

Histologic Findings

The livers of the recipient rats were removed and fixed in 10% buffered formalin, processed for light microscopy, and stained with hematoxylin and eosin (H-E) and with anti-insulin antibody to identify the grafted PIs.

Mixed Lymphocyte Reaction

For mixed lymphocyte reaction (MLR), splenic T cells were obtained by passing spleen cells through a nylon-wool fiber (Wako Pure Chemical, Osaka, Japan) column after incubation for 60 min at 37°C; 1 g of the nylon-wool fiber was prepared for 3×10^7 splenocytes to use as responders. Lymphocytes (3×10^6) were cocultured with 20 Gy-irradiated whole spleen cells (3×10^6: stimulator cells) in a total of 200 μl of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, and 50 μM of 2-mercaptoethanol (2-ME; Wako). The cultures were incubated at 37°C for 5 days in a humidified 5% CO_2 environment in 96-well flat-bottom microwell trays (Corning Glass Works, Corning, NY), and pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culture period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.

Assay for Generation of Cytotoxic T Lymphocytes

Cytotoxic T-lymphocyte (CTL) assays were evaluated by measuring the release of lactate dehydrogenase (LDH) from target cells using CytoTox 96 Non-Radioactive Cytotoxicity Assay Kits (Promega, Madison, WI), following the manufacturer's instructions. Briefly, splenocytes from F344 (RT1A^1), BN (RT1A^n), or ACI (August Copenhagen Irish; RT1A^a) rats were cultured with 2.5 mg/ml concanavalin A (Con A, Sigma, St. Louis, MO) for 3 days to prepare the Con A blastocytes as target cells. After washing, 3×10^5/well of Con A blastocytes were incubated for another 12 hr with or without 1×10^6/well of splenocytes from posttransplantation F344 or BN rats.

Statistical Analyses

Statistical analyses were performed using a two-tailed Student's *t*-test, except for graft survival rates. Statistical analyses of graft survival rates were performed using a log rank test.

RESULTS

Graft Survival

Because we have found that the injections of BMCs via the PV plus i.v. are effective in inducing persistent tolerance not only in chimeric-resistant MRL/lpr mice (*24*) but also in skin allografts of mice (*20, 25*) and pigs (*21*), we have attempted to examine the effect of PV injection of BMCs on tolerance induction using the PI transplantation system. To detect the

diabetic condition, nonfasting blood glucose levels were monitored every other day in F344 rats in which diabetes mellitus had been induced by streptozotocin. Plasma glucose levels in all recipient rats returned to normal within 24 hr after the PI transplantation (Fig. 1A). We monitored the graft survival by measuring nonfasting blood glucose levels. In the 8 Gy+PV+i.v., 8.5 Gy+i.v.+i.v., and 9 Gy+i.v.+i.v. groups, all grafts were rejected within 30 days. The 9 Gy+PV group showed a 70% graft survival rate 320 days after transplantation, whereas the 9 Gy+PV+i.v. group showed a 100% graft survived rate more than 1 year after the PI transplantation. All rats (10 of 10) in the 9 Gy+PV+i.v. group showed normoglycemia for more than 1 year. The graft survival rate in the 8.5 Gy+PV+i.v. group was 44% 150 days after transplantation (Fig. 1). In the 8.5 Gy+PV group, only one in seven cases accepted the PIs until day 185. These results suggest that simultaneous PV injection of BMCs induces tolerance to allogeneic PIs more easily than the i.v. injection of BMCs, and that the additional i.v. injection of BMCs maintains the tolerance induced by the PV injection of BMCs.

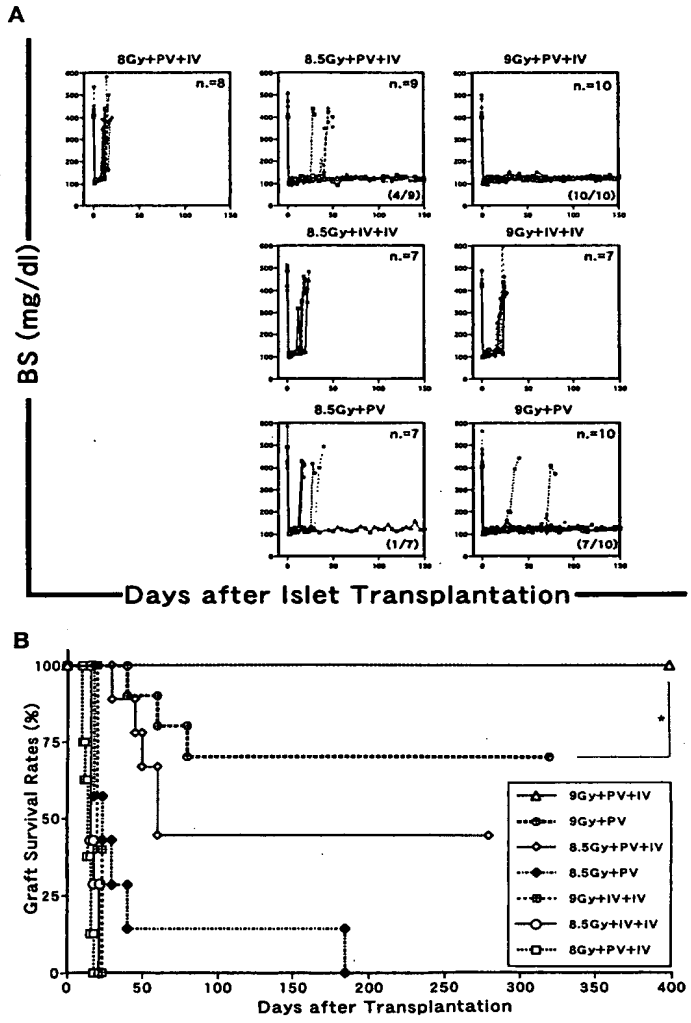

FIGURE 1. Graft survival of transplanted PIs. After irradiation, the PIs of BN rats were transplanted into F344 rats with BMCs of BN rats via the PV or i.v. Additional i.v. injection of BMCs from BN rats was performed in some groups, as described in *Materials and Methods*. Graft survival terms (A) and graft survival rates (B) in each experimental group are shown (*$P<0.01$). BS, blood sugar.

Histologic Findings

The livers of recipient rats were stained with H-E and with anti-insulin antibody to identify the grafted PIs. Grafted PIs were found in the 9 Gy+PV+i.v. group (60 days after transplantation; Fig. 2A), whereas disrupted islets, into which lymphocytes and macrophages had infiltrated, were seen in the livers of the 9 Gy+i.v.+i.v. group 30 days after transplantation (Fig. 2B). In the livers of the 9 Gy+PV+i.v. group, the grafted PIs were found even 200 days after the transplantation (Fig. 2C), although they were scattered throughout the liver in contrast with their distribution at 60 days after transplantation. The PIs grafted via the PV formed clusters in the liver for a short time, but they later became scattered throughout the liver. This seems to be related to the fact that the hepatocytes can proliferate, while the PI cells cannot.

Insulin was positively stained in the grafted PIs (Fig. 2D). In contrast, infiltrating mononuclear cells and fibrosis were seen in the livers of the 9 Gy+i.v.+i.v. group, and no insulin-positive PIs were found in the livers (data not shown). These results suggest that functional PIs exist in the livers of normoglycemic recipient rats, and that the grafted PIs are destroyed by immunologic mechanisms in the livers of hyperglycemic recipient rats.

Flow Cytometric Analyses of PBMCs

Chimerism was evaluated by flow cytometric analyses 90 days after transplantation using recipient PBMCs and anti-class I rat mAbs. The PBMCs in all graft-accepting rats showed the donor phenotype, although those of one graft-accepting rat in the 8.5 Gy+PV group partially showed the recipient phenotype, the level being 18.4% on day 90. Moreover, the transplanted PIs of this rat were rejected on day 185 when PBMCs showed only the recipient phenotype. The PBMCs in all the graft-rejecting rats showed the recipient phenotype (Fig. 3). However, all the PI-transplanted rats treated with 9 Gy+PV+i.v. survived more than 1 year, and their PBMCs were donor-type cells (>98%; Fig. 3). These results suggest that the existence of donor bone marrow-derived cells is crucial for successful transplantation of the PIs.

Intravenous Glucose Tolerance Tests and Serum Insulin Levels. As nonfasting blood glucose levels do not provide an accurate assessment of true regulation of glucose homeostasis in the PI graft-accepting recipients, i.v. glucose tolerance tests (IVGTTs) were performed and the serum insulin levels measured to evaluate the functions of the grafted PIs 150 days after transplantation. We compared the IVGTTs between the rats treated with 9 Gy+PV+i.v. and untreated control F344 rats (Fig. 4A). Fasting blood glucose levels were not significantly different between the graft-accepting rats (n=6) and untreated normal control rats (n=6). At 15 and 30 min after glucose administration, the PI-accepting rats showed a slightly higher blood glucose level than untreated normal control rats, although there was no significant difference. At 60 and 120 min after glucose administration, the blood glucose levels in the PI-accepting rats and untreated normal control rats returned to the levels before glucose administration and showed similar values.

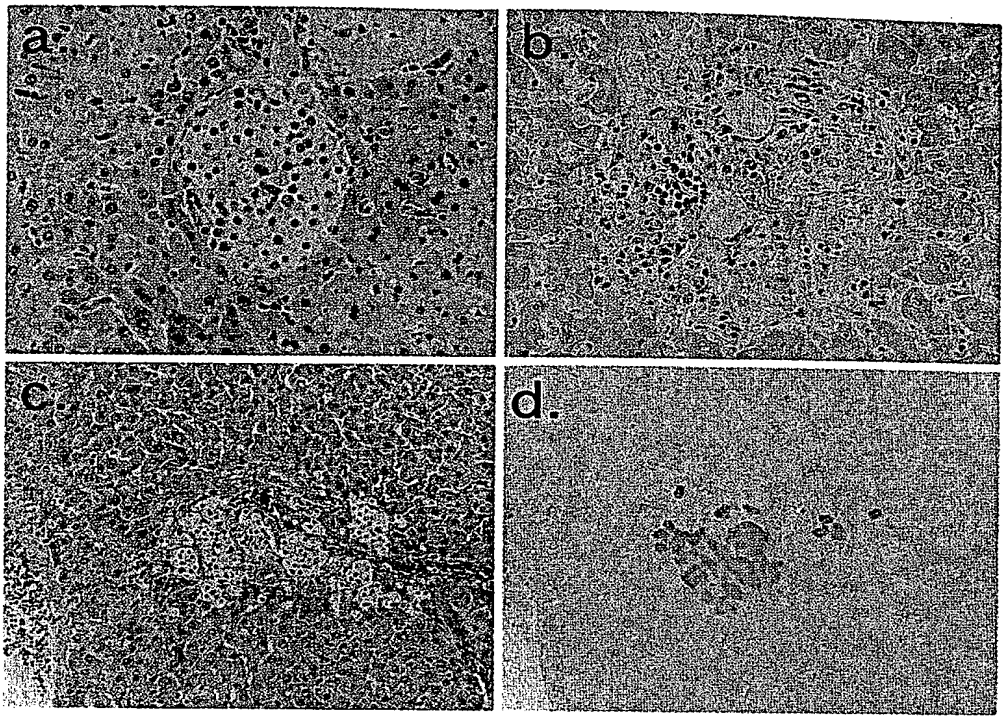

FIGURE 2. Histologic findings of transplanted PIs. PI-transplanted rats were killed 60 days after transplantation, followed by the histologic study of the livers, into which allogeneic PIs had been transplanted via the PV. Histologic examination was performed as described in *Materials and Methods*. The liver of a PI-transplanted rat with normoglycemia in nonfasting blood (a, H-E staining) and the liver of a PI-transplanted rat with hyperglycemia (b, H-E staining) are shown. PI-transplanted rats with normoglycemia were killed also 180 days after transplantation, and representative data on H-E staining (c) or immunohistologic staining for insulin (d) are shown.

We next measured the serum insulin levels in the groups of untreated normal control F344 rats (n=7), diabetes mellitus (DM)-induced nongrafting rats (n=7), PI-rejecting rats (n=7), and PI-accepting rats (n=12; Fig. 4B). The insulin levels in the group of DM-induced and PI-rejecting rats were significantly lower than those in the group of untreated normal rats. The insulin levels in PI-accepting rats were significantly higher than those in the PI-rejecting rats and were slightly lower than those in untreated normal rats, although there was no significant difference. These results suggest that the insulin levels of the DM rats do not recover owing to the destruction of beta cells, and that glucose homeostasis is maintained by the grafted PIs. The insulin levels in the rats treated with 9 Gy irradiation plus PV injection of only BMCs (without grafting PIs) followed by i.v. injection of BMCs after 5 days were similar to those of DM-induced rats (data not shown).

Mixed Lymphocyte Reaction

The splenic T cells of recipient rats that had received the PIs and BMCs from the donors were examined for their reactivity to the recipient, donor, and third-party alloanti-

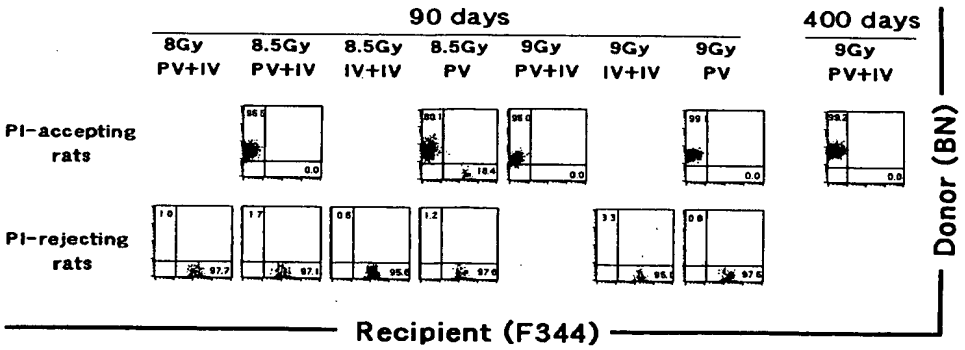

FIGURE 3. Fluorescence-activated cell sorter analyses using PBMCs of posttransplanted rats. The phenotypes of MHC class I in the PBMCs obtained from the PI-transplanted rats 90 days after various treatments (as indicated in this figure) or 400 days after the treatment with PV+i.v. were analyzed using a FACScan. Representative data in each experimental group are shown.

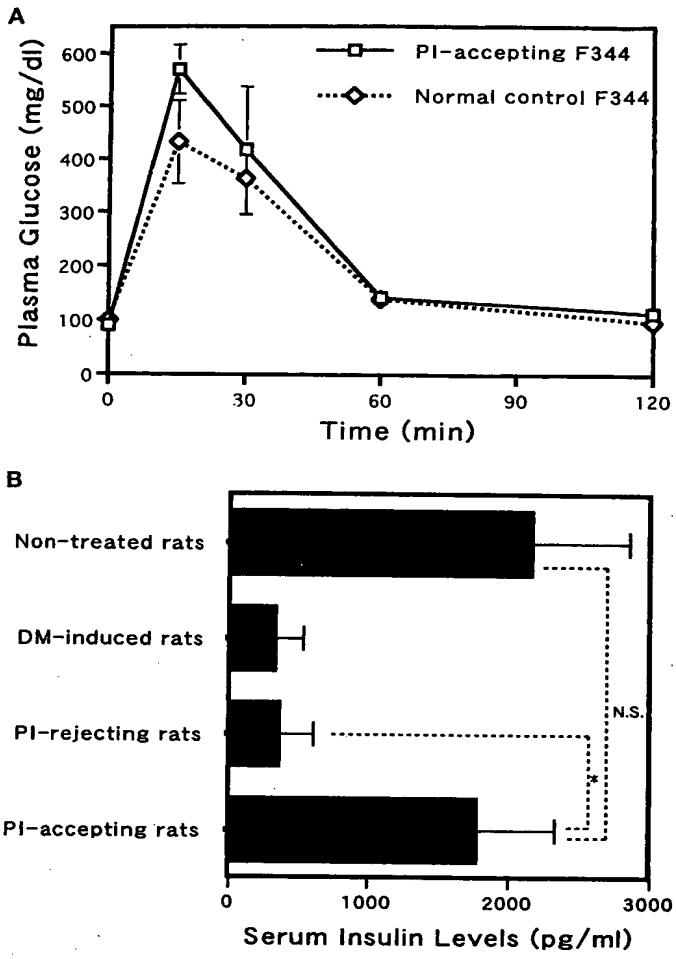

FIGURE 4. (A) IVGTTs of PI-transplanted rats in the 9 Gy+PV+i.v. group with normoglycemia in nonfasting blood were performed, and compared with those of untreated normal control rats. (B) Normal serum insulin levels of PI-accepting rats. Serum insulin levels were measured 150 days after PI transplantation, as described in *Materials and Methods* (*$P<0.005$, N.S., not significant).

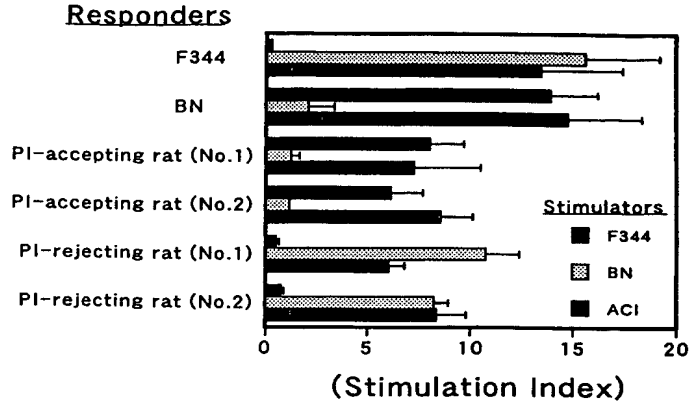

FIGURE 5. MLR in T cells from posttransplanted rats. MLR assays were performed as described in *Materials and Methods*. Splenocytes (3×10^7) as stimulators from untreated F344, BN, and ACI rats were cultured for 5 days with or without T cells (3×10^6) of posttransplanted F344, BN, or ACI rats. They were pulsed with 0.5 μCi [^{3}H]-thymidine per well for the last 20 hr of the culturing period. Stimulation indices were calculated by normalization to self-reactivity, which was near background incorporation in all cases.

gens using MLR assays 150 days after transplantation (Fig. 5). Untreated normal control F344 and BN rats showed normal proliferative responses to stimulator cells. The T cells of the graft-rejecting rats showed no response to the splenic cells of recipient F344 rats, although they did show responses to both donor and third-party MHC determinants. In contrast, the T cells of graft-accepting recipients, which were tolerant to donor alloantigens, showed similar proliferative responses to recipient stimulator cells as well as MHC-disparate third-party rat stimulator cells. Thus, the T cells of the rats with successful transplants responded to not only third-party cells but also host-type cells, although the graft-accepting recipients did not show any symptoms of graft-versus-host reaction (GvHR) for more than 1 year.

CTL Assays

Inasmuch as the graft-accepting rats showed the proliferative responses to host splenocytes in MLR assays but showed no symptoms of GvHR, we next performed the assays for the generation of CTLs to examine whether transplanted HSCs show cytotoxic effects on recipient cells in vitro. As shown in Figure 6, the splenocytes of F344 rats showed cytotoxic effects on both BN rat and third-party ACI rat cells, and the splenocytes of the donor BN rats showed cytotoxic effects on the recipient F344 rat cells. However, the graft-accepting rats showed no cytotoxic effects on not only donor rat (BN) cells but also recipient rat (F344) cells, although they showed a significant response to ACI (a third-party) rat cells. These results suggest that the transplanted donor HSCs do not injure the host cells even in vitro, although the T cells of the graft-accepting rats showed the proliferative responses to host cells in MLR assays.

DISCUSSION

Successful transplantation of tissues, cells, and organs between fully MHC-mismatched donor and recipient combinations has been dependent on the use of immunosuppressive agents to control acute and chronic rejection. Immunosuppressants have some toxic effects on various cells, which results in significant morbidity and mortality. It has been recognized that the induction of donor-specific tolerance by bone marrow chimerism can eliminate the problem of allograft rejection (*11–15*). Although bone marrow chimerism can successfully prevent even chronic graft rejection, the clinical application of the lethal conditioning approach to induce tolerance would be limited by the excessive toxicity associated with lethal conditioning.

The administration of insulin for the treatment of IDDM usually controls blood glucose and prevents lethal diabetic ketoacidosis. However, even frequent insulin treatments are not enough to prevent the high morbidity and mortality associated with IDDM (*26*). It has been reported that the transplantation of whole pancreas can maintain normal blood glucose levels and effectively control IDDM (*27*). The transplantation of the endocrine tissue alone (isolated PIs) is a

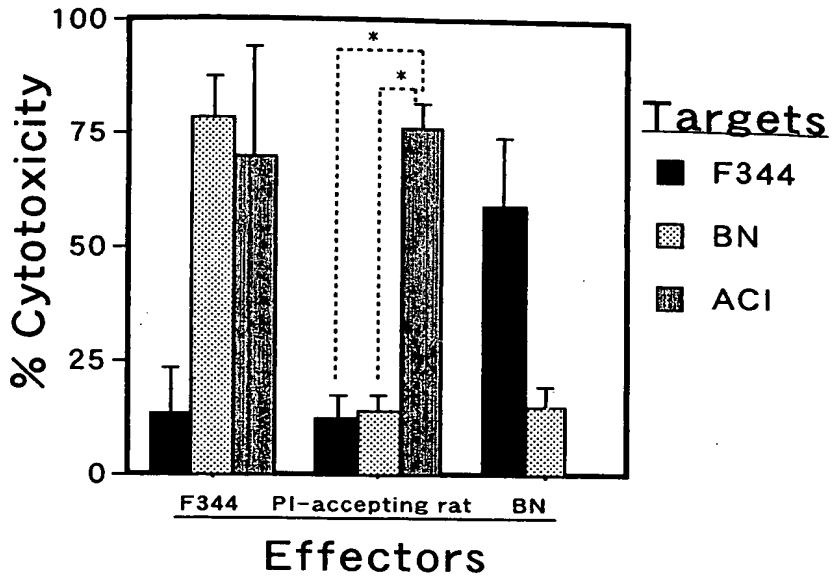

FIGURE 6. CTL assays. Con A blastocytes (3×10^5), which were prepared from splenocytes (1×10^6) of F344, BN, and ACI rats, were cultured for 12 hr with or without splenocytes from posttransplanted F344 or ACI rats, followed by measuring the levels of LDH in the wells using CytoTox 96 (*$P<0.05$).

more technically simple approach than whole pancreatic transplantation. It has also been reported that the survival term of the grafts in the transplantation of PIs is short owing to their high antigenicity and their high sensitivity to graft rejection (*28*).

It has been reported that systemically draining grafts (such as PI grafts under the renal capsule) directly secrete insulin into the systemic circulation and induce hyperinsulinemia (*29*). Therefore, the transplantation of PIs via the PV (PV drainage) is a more physiologic approach than systemic drainage (*5, 8*), and intrahepatic PI transplantation is easily performed. Clinically, PV injection is easily carried out under echographically guided portal puncture with local anesthesia. We have previously found that the PV administration of allogeneic cells can induce donor-specific tolerance across MHC barriers (*18*), and that the injection of BMCs via the PV followed by transient usage of an immunosuppressant without irradiation can induce persistent tolerance in skin allografts (*20, 21*). In the present study, we have found a strategy for long-term acceptance of PIs with normal functions without recourse to immunosuppressants: a sublethal irradiation dose (9 Gy) plus simultaneous injection of BMCs and PIs via the PV followed by i.v. injection of BMCs. The 9 Gy+PV+i.v. group showed normal glucose levels and a 100% survival rate, and did not develop IDDM by 400 days after transplantation (Fig. 1), whereas the 9 Gy+i.v.+i.v. group showed high glucose levels owing to the rejection of the PIs (Fig. 1A). The PBMCs in all but one graft-accepting rat showed the donor phenotype. The exception was in the 8.5 Gy+PV group; 18.4% recipient phenotype was detected in the PBMCs in this rat at 90 days after transplantation, indicating that mixed chimerism existed at that time. The PI graft of this rat was rejected 185 days after transplantation, at which time no donor cells were detected in the PBMCs. The mechanism underlying the loss of mixed chimerism has not been elucidated, but the loss may reflect the presence of MHC restriction (preference) between HSCs and stromal cells, as we previously described (*30, 31*). Therefore, the complete replacement of hemopoietic cells by donor cells (instead of mixed chimeras) is crucial to the induction of persistent tolerance, as we have previously shown in lethally irradiated mice (*1, 11–13, 24*). We have previously used T-cell–depleted BMCs for allogeneic bone marrow transplantation (BMT). However, we have recently used whole BMCs, which contain a small number of T cells (<1%). The T cells present in the bone marrow were found not to induce GvHR, but to facilitate bone marrow engraftment (prevent HvGR) (*32, 33*), even when sublethal irradiation doses were used for allogeneic BMT (*34*) and organ transplantation (*25*). It should be noted that sublethal irradiation (7 Gy for mice (*25*) and 9 Gy for rats in the present study) induces fully allogeneic chimerism (>98%) for more than 1 year without evidence of either GvHR or HvGR.

It is thought that IDDM is an organ-specific autoimmune disease, which is characterized by the destruction of insulin-producing beta cells by autoimmune mechanisms (*1*). The nonobese diabetic (NOD) mouse is a well-known animal model for IDDM. We have previously shown that allogeneic BMT can prevent and treat insulitis (*1*), and that allogeneic BMT plus fetal pancreas grafts can treat overt diabetes in NOD mice (*12*). However, we have found that in (BALB/c+NOD→NOD) chimeric mice, NOD hemopoietic cells become dominant, which results in the development of IDDM (*35*), because the abnormal HSCs of autoimmune-prone mice are more resilient than normal HSCs, as we previously described (*36*). These findings suggest that allogeneic BMT instead of mixed allogeneic BMT should be carried out in conjunction with organ transplantation.

Hemopoietic cells from PI-accepting rats showed the proliferative response to host cells in MLR assays in vitro. However, they showed no cytotoxic effects on host cells not only in vivo (no GVHR) but also in CTL assays in vitro. This split tolerance, as previously described by Sprent et al. (*37*), is interesting for analyzing the mechanisms underlying tolerance induction. It is conceivable that some suppressor mechanisms that inhibit the functions of cytotoxic effector cells

against the host are involved. We are now in the process of examining regulatory and suppressor cells in our system.

In conclusion, we have demonstrated that the combination of PV plus i.v. injections of BMCs is effective in inducing donor-specific tolerance across MHC barriers, even when the irradiation dose is reduced to 9 Gy (sublethal dose). This strategy (9 Gy+PV+i.v.) leads to long-term acceptance of PIs with normal functions, enabling the treatment of diabetes mellitus without recourse to immunosuppressants.

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Long-Term (>1 year) Analyses of Chimerism and Tolerance in Mixed Allogeneic Chimeric Mice Using Normal Mouse Combinations

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ABSTRACT

We examined the induction of tolerance using pancreas allografts over the long term (>1 year) in mice for the human application of mixed allogeneic bone marrow transplantation (BMT). T cell-depleted BM cells (BMCs) of C57BL/6 (B6) and C3H/He (C3H) mice were transplanted at various ratios into lethally irradiated B6 mice. The percentages of C3H cells in the chimeric mice gradually decreased, finally declining to only a small percentage, except when the ratio of donor to recipient BMCs was 100:1. However, despite the marked decreases in C3H-type cells, all the pancreas allografts of C3H mice were accepted when more than 1% C3H cells were detected in the peripheral blood. To examine the relationships between percentages of transplanted donor cells and acceptance of pancreas allografts, various percentages of donor and recipient BMCs (5% to 30%) were transplanted. It was found that more than 10% donor cells were necessary for the pancreas allografts to be accepted. In vitro assays for mixed lymphocyte reaction and generation of cytotoxic T-lymphocytes revealed that spleen cells in chimeric mice accepting pancreas allografts are tolerant to both host-type and donor-type major histocompatibility complex (MHC) determinants, but show a vigorous responsiveness to third-party MHC determinants. Since donor-type hemopoietic stem cells (HSCs) were detected in the BM and the liver of the chimeric mice, donor-derived HSCs and donor-derived hematolymphoid cells are responsible for the induction of tolerance. It should be noted that the percentage of donor-type HSCs is higher in the liver (6.2%) than in the BM (0.9%). *Stem Cells 2000;18:273-280*

INTRODUCTION

In humans, organ allografts require the use of immunosuppressants to prevent graft rejection. In mice, we have previously shown that allografts of organs such as the liver and pancreas, in conjunction with allogeneic bone marrow transplantation (BMT) from the same major histocompatibily complex (MHC) donors, can induce permanent tolerance without using immunosuppressants [1, 2]. *Ildstad* and *Sachs* have established a system of mixed allogeneic chimerism by carrying out mixed allogeneic BMT and have demonstrated that mixed allogeneic BMT can be used for organ transplantation [3]. Mixed allogeneic chimerism has several advantages over fully allogeneic chimerism. The presence of syngeneic (or autologous) bone marrow cells (BMCs) appears to provide the necessary cells to overcome the impaired immunologic functions and prevent the graft-versus-host-disease (GVHD) observed in fully allogeneic chimeras, while the allogeneic BM elements appear to be responsible for the induction of donor-specific tolerance. *Starzl et al.* have found in humans that there are some cases in which liver allografts survive without using immunosuppressants, and that, in such cases, a small number of hemopoietic cells derived from the transplanted organs are detected: they described this as microchimerism [4]. In mice, mixed allogeneic chimerism can be achieved by carrying out mixed allogeneic BMT. In this condition, donor-specific tolerance can be induced. However, for human application to organ transplantation, long-term observation using allografts of organs other than the skin is necessary, since we have very recently found that the skin is not rejected after donor-derived Langerhans' cells have been replaced by

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host-derived Langerhans' cells, even when host-derived hematolymphoid cells become dominant (manuscript in preparation). In addition, we have found that the pancreas is more immunogeneic than the skin (manuscript in preparation), although it has been thought that the skin is the most immunogeneic tissue. Therefore, we examine the induction of tolerance, chimerism, and pancreas allograft acceptance in normal mouse combinations over a long term (>480 days after transplantation). In the present study, we show that mixed allogeneic BMT can be used for organ allografts, although the number of allogeneic hematolymphoid cells gradually decreases.

Materials and Methods

Mice

Female C57BL/6J (B6: H-2^b), C3H/HeN (C3H: H-2^k), and BALB/c (H-2^d) mice (five to eight weeks old) were purchased from SLC (Shizuoka, Japan), and raised at the Kansai Medical Animal Care Center.

Mixed Allogeneic BMT ([B6 + C3H]→B6)

Mixed allogeneic chimeric mice were prepared as previously described [3]. Briefly, inbred B6 female recipient mice were lethally irradiated with 10 Gy from a ^{137}Cs source (Gammacell 40 Exactor, Nordion International Inc.; Kanta, Ontario; http://www.MDSNordion.com). The BM was flushed from the femurs of the B6 and C3H donor mice with RPMI 1640 using a 23-gauge needle. The BMCs were gently resuspended with a 21-gauge needle, and the suspension was filtered through a nylon mesh. The BMCs were then washed at 1,500 rpm for 5 min, resuspended in RPMI, and counted. T cells in the BMCs were depleted using anti-Thy1.2 monoclonal antibody (mAb) (F7D5, Olac; Bicester, England) at 4°C for 30 min. They were then washed and resuspended in guinea pig complement at a 1/16 dilution in RPMI (1×10^7 cells/ml in diluted guinea pig complement) at 37°C for 40 min. The BMCs were then washed twice and resuspended in RPMI at an appropriate concentration for injection of 0.5 ml of final volume per mouse. The recipient mice were reconstituted within 12 to 24 h after irradiation with 1×10^7 BMCs mixed at various ratios (B6: C3H = 2:1, 1:1, 1:3, 1:10, or 1:100, and B6 or C3H only). In some experiments, the recipient mice were reconstituted in the ratios of 5% (18:1), 10% (9:1), 20% (4:1), and 30% (7:3), respectively.

All recipients were evaluated for the presence of clinical GVHD as manifested by weight loss, alopecia, ruffled fur, diarrhea and a decreased level of activity associated with a "hunched over" appearance. In addition, histological evidence for GVHD was evaluated using samples of the skin, liver, intestine and hematolymphoid organs (the spleen, lymph nodes, thymus and BM).

Transplantation of Fetal Pancreas Tissue

The procedure was as described previously [5]. Briefly, recipients were anesthetized with an i.p. injection of somnopentyl (0.1mg/g body weight). Light ether anesthesia was used, if necessary, during the operation. A vertical incision was made in the lumbar region, and the underlying kidney gently pulled out of the abdomen.

A longitudinal incision was made in the renal capsule. The edge of the incised capsule was lifted up with fine forceps, and the fetal pancreas grafts placed under the capsule, and pushed away from the incision. The fetal pancreas was used because it contains more islets, but less exocrine glands, than the adult pancreas. The kidney was replaced within the peritoneal cavity and abdominal muscles, and the skin incision closed with silk sutures. It required about 10 min for a single engraftment.

Cell Preparation

Peripheral blood (PB) was collected into heparinized plastic vials from the orbital cavity. After mixing, the suspension was layered over 1.5 ml of room temperature lymphocyte separation medium (Lympholyte-M; Cedarlane; Hornby, Ontario; http://www.cedarlanelabs.com) and centrifuged at 3,000 rpm for 30 min at 23°C. The lymphocyte layer was aspirated from the saline-Lympholyte-M interface and washed with medium.

BMCs were collected from the femurs of recipient mice, as previously described [5]. The spleen and lymph nodes were gently teased on a fine steel mesh, and cell suspensions washed twice in RPMI-1640 medium (Nissui; Tokyo, Japan; http://www.nissui.co.jp/top.html), and finally suspended in medium containing 10% fetal calf serum (FCS) (HyClone Laboratories; Logan, UT; http://www.hyclone.com).

Hepatic mononuclear cells (MNCs) were obtained as follows: the liver was perfused in situ via the portal vein with 10 ml of Dulbecco's phosphate-buffered saline (PBS) and 10 ml of prewarmed (38°C) PBS(-) containing 150 U/ml Type IV collagenase (Sigma Chemical Co.; St. Louis, MO; http://www.sigma-aldrich.com). The liver was removed and cut into small pieces. The tissue was transferred into a 50-ml tube, dispersed by pipetting, and added to 40 ml of PBS containing 2% FCS. The cell suspension was centrifuged at 35× *g* for 1 min at 4°C to remove tissue debris and parenchymal cells. The hepatic MNCs in the supernatant were washed three times at 250× *g* for 5 min. The hepatic MNCs in the pellet of the last centrifugation were suspended in 2 ml of 31.5% Percoll solution (Pharmacia; Uppsala, Sweden; http://www.pnu.com), layered onto 2 ml of 70% Percoll solution in a 15-ml tube, and covered with 2 ml of PBS. After the centrifugation at 450× *g* for 20 min, the hepatic MNCs in the lower interface were

removed and washed twice. The recovery for hepatic MNCs was about 31%, and the contamination of PB MNCs was less than 1%.

Flow Cytometry

Fluorescein isothiocyanate (FITC)-coupled anti-H-2K^k (030-39F) and H-2K^b (030-11F) mAbs from Meiji Institute of Health Service (Odawara, Japan) and phycoerythrin (PE)-coupled anti-H-2K^b mAb from PharMingen (San Diego, CA; http://www.pharmingen.com) were used for determining the percentage of cells bearing MHC class I (H-2K^b and H-2K^k) surface markers in the PB lymphocytes, BM, spleen, lymph nodes, and liver. PE-coupled anti-CD4, CD8, B220, Mac-1, Gr-1, and CD71 mAbs from PharMingen were used for characterizing the donor-derived BM and liver MNCs. mAbs against erythroid lineage cells (TER119) were kindly donated by *T. Kina* (Chest Disease Institute; Kyoto University, Kyoto, Japan; http://www.Kyoto-u.ac.jp). The cells were suspended in PBS containing 2% FCS plus sodium azide, then incubated on ice with the appropriate mAbs for 30 min and analyzed by flow cytometry on FACScan (Becton Dickinson & Co.; Mountain View, CA; http://www.bd.com) equipped with logarithmic scales.

Mixed Lymphocyte Reaction (MLR)

Triplicate cultures from four chimeric mice and four control mice were set up in round-bottom 96-well microwell trays (Corning Inc.; Corning, NY; http://www.corning.com). Each well contained 2×10^5 responder cells and 10^5 stimulator cells in a total of 0.2 ml of RPMI 1640 medium supplemented with 2mM L-glutamine, penicillin (100 units/ml), streptomycin (100 mg/ml) (Sigma-Aldrich; St. Louis, MO), 10% heat-inactivated FCS, and 50 mM 2-mercaptoethanol (2-ME; Wako; Osaka, Japan). Stimulator cells were irradiated with 20 Gy. The cultures were incubated for 96 h in a humidified 5% CO_2 atmosphere. [^{3}H] thymidine (0.5 mCi) was introduced during the last 4 h of the culture period. [^{3}H] incorporated into trichloroacetic acid-insoluble materials was measured using a liquid scintillation counter.

Generation of Cytotoxic T-Lymphocytes (CTLs)

Responder cells (7.5×10^6) and stimulater cells (2.5×10^6) were cocultured in RPMI 1640 medium containing 10% heat-inactivated FCS, supplemented with 50 mM 2-ME. Cultures were incubated for five days at 37°C in a 5% CO_2 atmosphere. Cells of the cell lines (P815 [H-2^d], EL-4 [H-2^b] and X5563 [H-2^k]) were used as target cells. These cells were labeled by incubation for 1 h at 37°C with 100 mCi of Na251CrO4 (NEN Life Science Products Inc.; Boston, MA; http://w.nenlifesci.com). After washing three times, labeled cells (5×10^4) were mixed with effector cells in 100 ml of RPMI 1640 medium in round-bottom microwells and incubated at 37°C in 5% CO_2 for 4 h. The Titertek supernatant system was used for determination of released radioactivity of ^{51}Cr.

Percent-specific lysis was calculated as ([experimental release-spontaneous release]/[maximal release-spontaneous release]) × 100. In the analyses, spleen cells were pooled from mice, and the analyses were performed in triplicate.

Histology

Recipient mice were sacrificed each month after engraftment. The grafts were easily identified as a rounded white swelling on the surface of the kidney. The acceptance and growth of the grafts was assessed using a dissecting microscope, and the kidneys from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for the histological examination.

The data shown in the figures and tables are representative data since reproducible results were obtained.

Results

Analyses of Chimerism

Chimerism was analyzed by flow cytometry using FITC-conjugated anti-class I (H-2^b and H-2^k) mAbs. As shown in Figure 1, mixed chimerism was observed in almost all chimeric mice except the mice reconstituted at a host:donor ratio of 1:100. Donor-type T cells, B cells and macrophages were detected (data not shown). Since there was no significant difference between the spleen, lymph nodes and PB, the degree of chimerism was shown in the PB. As shown in Figure 1, donor-type cells (H-2K^k) gradually decreased in all mice except for the mice reconstituted with a ratio of 1:100. However, it should be noted that donor-type cells did not completely disappear. Even at 15 months, 38.0%, 12.0%, 8.2% and 6.2% of donor-type cells were detected in mice reconstituted with the ratios of 1:10, 1:3, 1:1 and 2:1, respectively. Although observations were continued for 16 months, no symptoms of GVHD were clinically or histologically observed in the mixed allogeneic chimeric mice. Complete replacement with donor-type cells was observed in mice reconstituted at a ratio of 1:100.

Graft Acceptance

The pancreas tissues transplanted under the capsules of the kidneys in the recipient mice were histologically examined at various time points. As shown in Figure 2, the grafts were accepted by the recipient mice with established mixed chimerism, but rejected in mice without mixed chimerism. Even in mice with a markedly decreased percentage of donor-type cells, no destruction of grafted pancreatic tissue was observed when more than 1% of donor cells were detected in the PB. Immunohistochemical studies revealed

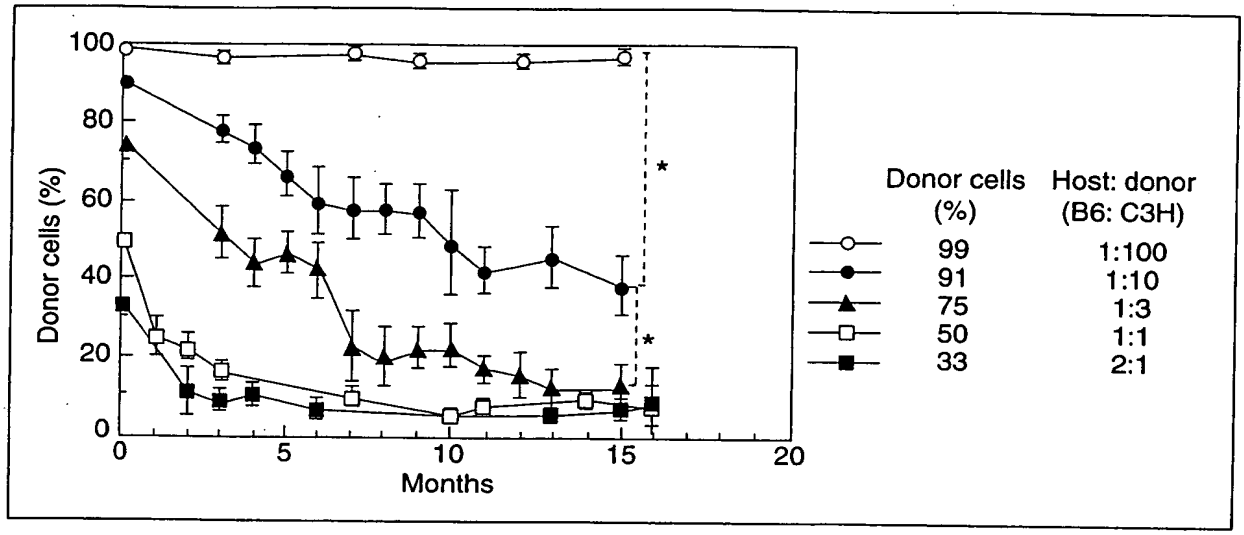

Figure 1. Percentages of donor (H-2K^k)-derived cells in the PB of mixed chimeras by two-color FACS analyses. B6 hosts were lethally irradiated (10 Gy) and then reconstituted with a mixture (total 1 × 10^7) of T cell-depleted syngeneic and allogeneic BMCs with various proportions, as described in Materials and Methods. PB was collected from the mice in each group every month after treatment. The groups of 1:100, 1:10, 1:3, 1:1 and 2:1 consisted of 10, 15, 15, 13, and 7 mice, respectively. Statistical analyses were performed by Mann-Whitney U-test: $p < 0.005$, 1:10 versus 1:100 and 1:3.

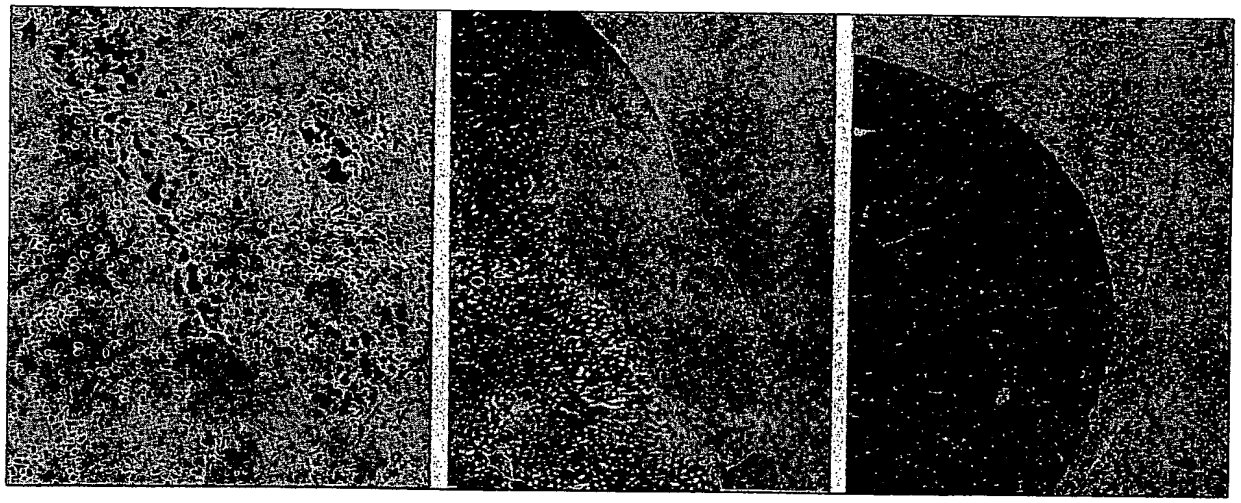

Figure 2. Histological findings of the grafts. Recipients were transplantated with the fetal pancreas under the capsule of the kidney. The mice were sacrificed 3, 6, and 12 months after transplantation, and the grafts were stained with hematoxylin and eosin. In this figure, representative pictures in mice (one year after transplantation) reconstituted with a 1:3 ratio are shown; the mixed chimeric mouse (B6 + C3H[1:3]→C3H) accepts C3H pancreatic tissue (islets) with insulin-producing cells (brown) (left), whereas such a chimeric mouse rejects the third-party (BALB/c) pancreas tissue (middle). (B6→B6) mouse rejects C3H pancreas tissue (right). The rejected pancreatic tissues were replaced by fibrous tissue (middle) or adipose tissue (right) within one month after transplantation.

the presence of insulin-producing cells (brown) in the engrafted islets (Fig. 2 left).

Induction of Tolerance

The next step was to examine whether donor-specific tolerance is induced in mixed allogeneic chimeric mice using MLR and CTL assays.

MLR was performed to examine the induction of tolerance. Representative data are shown in Figure 3. Twelve months after transplantation, 97.13%, 45.8% and 14.3% of donor cells were detected in mice reconstituted with the ratios of 1:100, 1:10, and 1:3, respectively. All these chimeric mice showed low responses to both donor- and host-type stimulators, although they showed significantly

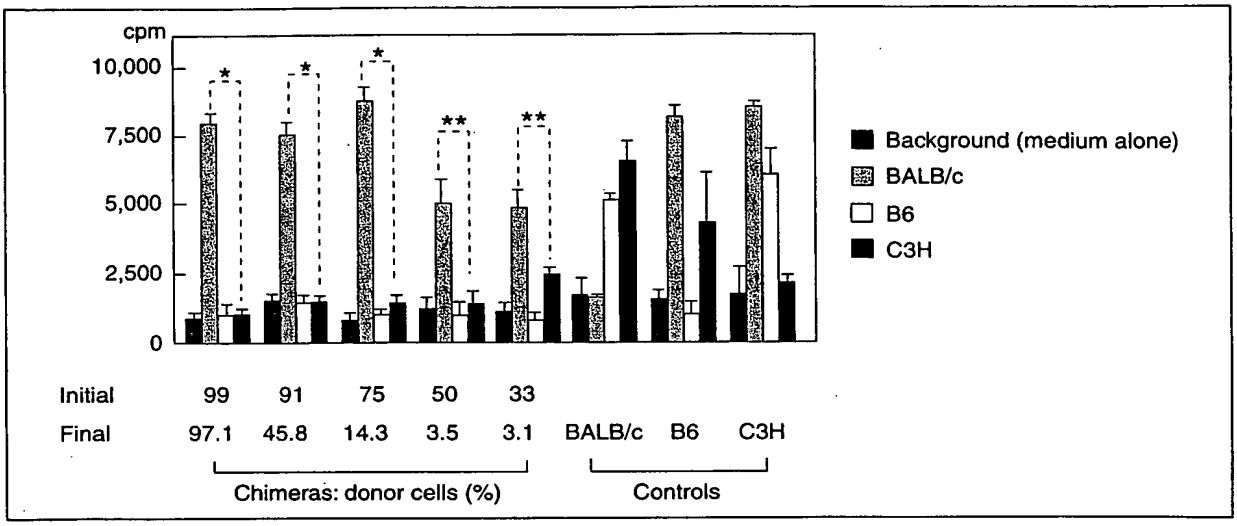

Figure 3. MLR of spleen cells in B6, C3H, BALB/c, and ([B6 + C3H]→B6) chimeric mice one year after treatment. More than three experiments were carried out, and reproducible results were obtained. Representative data are therefore shown. Chimeric mice respond only to third-party (BALB/c) stimulator. Asterisks represent p *values of responses to donor-type stimulators versus third party by* t*-test.* *p < *0.0001,* **p < *0.005.*

high responses to the third-party (BALB/c) cells (Fig. 3). Moreover, when 3.5% and 3.1% of donor cells were detected 12 months after transplantation in mice reconstituted at ratios of 1:1 and 2:1, these chimeric mice showed significantly high responses to the third-party (BALB/c) cells, whereas they showed low responses to both donor- and host-type stimulators (Fig. 3).

Table 1. Cytotoxic T-lymphocytes from spleen cells of chimeric mice are tolerant to both host-type and donor-type MHC determinants

Mouse	% specific lysis (E/T = 6)[a]		
	P815 ($H\text{-}2^d$)	EL4 ($H\text{-}2^b$)	BW5147 ($H\text{-}2^k$)
BALB/c ($H\text{-}2^d$)	0	43	79
C57BL/6 ($H\text{-}2^b$)	76	2	75
C3H/HeN ($H\text{-}2^k$)	81	48	2
(B6→B6)	75	2	76
(B6:C3H[1:100]→B6)	63	1	1*
(B6:C3H[1:10]→B6)	80	2	2*
(B6:C3H[1:3]→B6)	51	1	1*
(B6:C3H[1:1]→B6)	33	0	1*
(B6:C3H[2:1]→B6)	32	0	8*

[a]Spontaneous releases and maximum releases were 1,594 and 26,204 cpm in P815, 1,944 and 29,826 cpm in EL-4, and 1,345 and 18,515 cpm in BW 5147, respectively.

**p* value of cytotoxic activity to donor-type versus third party by *t*-test: $p < 0.0001$.

Similar results were obtained in CTL assays (Table 1). The spleen cells of the chimeric mice showed cytotoxic activity to third-party ($H\text{-}2^d$) cells, but not to host-type or donor-type cells.

Critical Doses of Donor Cells for Allograft Acceptance

To determine the critical doses of initially transplanted donor cells for allograft acceptance, we next carried out BMT using various ratios of donor and recipient BM cells; the ratios of donor BM were adjusted to 5%, 10%, 20%, or 30%. In addition, pancreatic tissue transplantation was performed at the same time. As shown in Table 2, the grafts were accepted by the recipient mice reconstituted with 10%, 20%, or 30% donor BM cells, but not 5% donor BM cells. Mixed chimerism was observed in recipient mice reconstituted with 10%, 20% or 30% donor BM. The induction of tolerance in these mice was examined by MLR. As shown in Figure 4, chimeric mice reconstituted with 5% donor BM cells, in which the grafts had been rejected, showed high responses to C3H mouse MHC determinants, while chimeric mice reconstituted with 10%, 20%, and 30% donor BM cells, in which the grafts were accepted, showed low responses to C3H mouse MHC determinants. From these results, it can be concluded that the presence of chimerism in the PB is useful for evaluating the induction of tolerance; it seems that more than 10% donor BM cells are initially necessary to prevent the rejection of donor pancreas tissue.

Table 2. Relationship between acceptance of donor pancreatic grafts and percentages of initially transplanted donor cells in chimeric mice[a]

Initial donor (C3H) cells (%)	After six mon		
	Mouse *n*	Graft	C3H (%)
5	1	–	0
	2	–	0
	3	–	0.1
10	4	+	1.5
	5	+	3.8
	6	+	2.6
20	7	+	5.8
	8	+	8.2
	9	+	23.9
30	10	+	11.0
	11	+	12.1

[a]Chimeric mice were perpared as described in **Materials and Methods**. Six months after BMT, the graft acceptance was examined, and the percentages of the donor cells in the peripheral blood were analyzed using a FACS.

Allogeneic Hemopoietic Stem Cells (HSCs) in the BM and Liver

Since donor-type T cells, B cells, and macrophages are observed in the BM and liver of the recipient mice for an extended period after transplantation, it is conceivable that donor-type HSCs are present in the recipient mice. We therefore analyzed whether donor-type HSCs are present in the BM and liver using a fluorescence-activated cell sorter (FACS), as previously described [6]. As shown in Figure 5, $Lin^-/CD71^-/H\text{-}2^{high}$ cells, which are pluripotent HSCs, as previously described [6-8], were observed in the BM and liver; it should be noted that the percentage of donor HSCs is higher in the liver (6.2%) than in BM (0.0%).

Discussion

A number of approaches to achieve allogeneic chimerism with lethal or nonlethal conditions have been reported in rodent models [3, 9-11]. Mixed allogeneic chimerism has several advantages over fully allogeneic chimerism. The presence of syngeneic (or autologous) BMCs appears to provide the necessary cells to overcome the impaired immunologic functions and prevent the GVHD observed in fully allogeneic chimeras [12], while the allogeneic BM elements appear responsible for the induction of donor-specific tolerance. In mixed chimeras, primary antibody responses to T cell-dependent antigens are completely restored, since T cells can cooperate with B cells and antigen-presenting cells. Therefore, mixed allogeneic chimerism may be a useful approach to induce tolerance for solid organ and cellular grafts.

Ildstad and *Sachs* were the first to establish a system of mixed allogeneic chimerism which can be used for organ transplantation [3]. Using skin allografts, they have shown that persistent tolerance can be induced until 380 days by carrying out mixed allogeneic BMT. In the present study using pancreas allografts, we have shown that persistent tolerance can be maintained for more than one year (480 days) after mixed allogeneic BMT, although the percentages of donor-type (C3H) cells gradually decrease (Fig. 1). This decrease in the percentages of donor (C3H)-type cells can be explained by MHC restriction between HSCs and

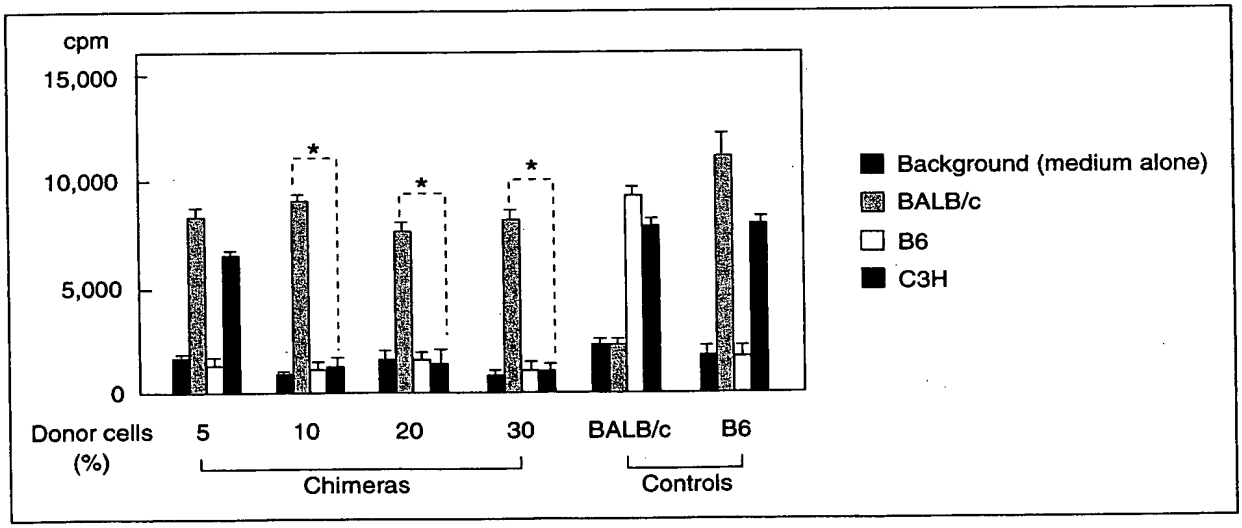

Figure 4. Relationship between percentages of donor cells and responsiveness in MLR six months after treatment. *The spleen cells of mixed chimeric mice with 5% of donor cells show a high responsiveness to donor (C3H)-type MHC determinants, although the spleen cells of mixed chimeric mice with more than an initial 10% of donor cells show low responsiveness to not only host-type but also donor-type MHC determinants. Asterisks represent* p *values of responses to donor-type stimulators versus third-party by* t*-test:* $*p < 0.001$.

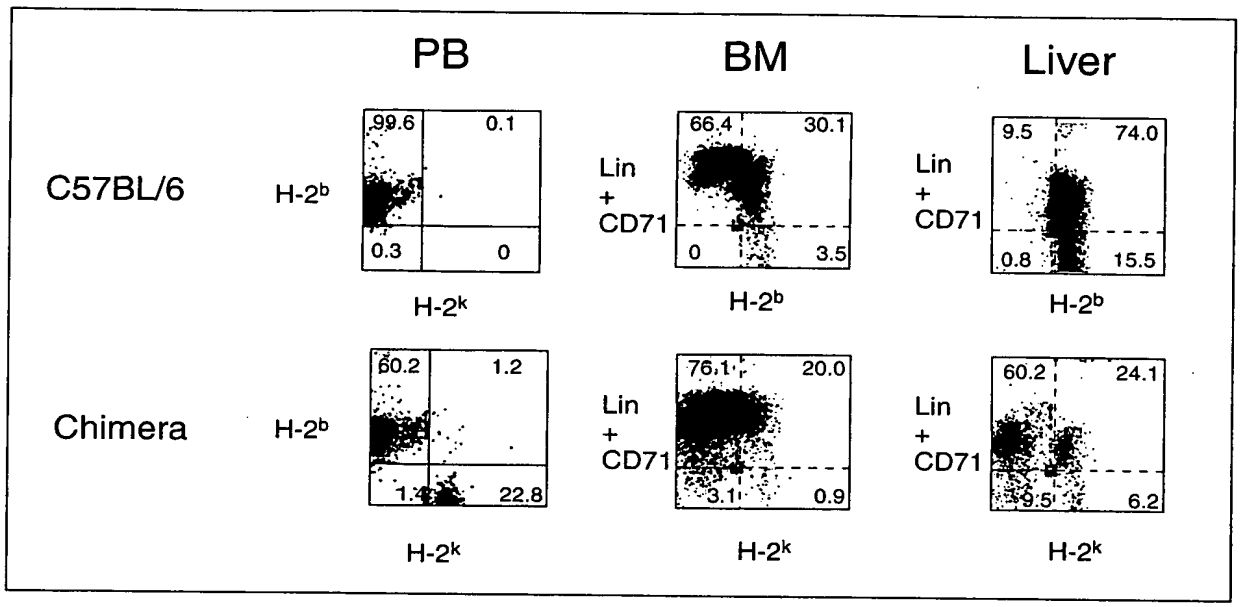

Figure 5. The characterization of the donor-derived BM and liver MNCs by flow cytometry three months after treatment. BM and liver MNCs of chimeric mice and B/6 mice (control) were stained with H-2K^k, H-2K^b, CD71 mAbs, and lineage markers (CD4, CD8, B220, Mac-1, TER119, Gr-1). The populations of lineages CD71$^-$ and H-2K^{high} are HSC-enriched. Chimeric mice (four mice) were analyzed, and similar results were obtained. Therefore, representative data are shown in this figure.

stromal cells; in the ([B6 + C3H]→B6) chimeric mice, B6 HSCs should show a better proliferative response than C3H HSCs, as we have previously shown [13]. It should be noted that all the pancreas allografts are accepted when more than 1% of allogeneic cells are detected in the PB of the recipients. To further examine the relationship between percentages of initially transplanted donor cells and acceptance of pancreatic allografts, we carried out mixed allogeneic BMT using donor BM of decreased percentages (5% to 30%). Table 2 shows that more than 10% of donor cells are necessary to prevent graft rejection. It has been reported that recipient mice with >30% chimerism accept skin allografts in an MHC class II-disparate combination, although recipients with <10% chimerism show prolonged skin graft survival but finally reject skins [14]. It is conceivable that the difference between the data of *Taniguchi et al.* and ours is due to the grafts of different organs (the skin and pancreas) and different mouse combinations (only class II-disparate combination in the former and both class I- and class II-disparate combinations in the latter).

In the present study, we have shown that pancreas allografts are accepted by the recipient mice with establishment of mixed chimerism in the PB despite marked decreases in donor-type cells (Figs. 1 and 2; Table 2). MLR (Fig. 3) and CTL (Table 1) assays indicate the induction of systemic tolerance in these mice. Mechanisms underlying tolerance induction include clonal deletion [15, 16], anergy [17], and suppression [18, 19]. In the present study, we have demonstrated the presence of donor-derived allogeneic HSCs in the BM and liver (Fig. 4). Since we have previously found that donor HSCs trapped in the liver induce clonal anergy in the recipient CD8$^+$ CTLs [20], it is certain that clonal anergy is involved in tolerance induction in this mixed allogeneic chimerism. Although we have not examined the clonal deletion mechanism in this system, it is also conceivable that clonal deletion, not only in the thymus but also in the periphery, is involved in this system, since *Zavazava et al.* have recently demonstrated that soluble MHC class I molecules induce apoptosis in alloreactive CTLs [21]. Suppressor mechanisms appear to be involved in the establishment of tolerance induction even in mixed allogeneic BMT. Suppressor cells include suppressor T cells (CD8$^+$ cells), natural killer cells, and natural suppressor (NS) cells; we have previously found that NS cells belong to HSCs in the cycling phase in the BM [22].

The skin and pancreas are candidates to examine functional tolerance in vivo, since they are highly antigeneic and very sensitive to rejection [7]. *Starzl et al.* have found in humans that there are some cases in which liver allografts survive without using immunosuppressants, and that, in such cases, a small number of donor-derived hemopoietic cells are detected. We have very recently established the method for organ allografts by injecting allogeneic hemopoietic cells (including HSCs) from the portal vein;

the recipient mice show microchimerism [17], as shown in the present study. It should be noted that the percentage of donor-type HSCs is higher in the liver (6.2%) than in the BM (0.9%) (Fig. 5). We have previously found that HSCs trapped in the liver induce anergy of recipient CTL2 [20]. We are in the process of analyzing the exact mechanisms underlying tolerance induction in microchimerism.

In summary, we report here that long-term pancreatic allograft survival over a one-year period can be reliably achieved in MHC-disparate allogeneic donor and recipient combinations, although donor-type cells gradually decrease. The tolerance was highly MHC-specific, as evidenced by MLR and CTL assays.

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Development of Insulin-Dependent Diabetes Mellitus in [(NOD + BALB/c) → NOD] Mixed Allogeneic Bone Marrow Chimeras

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Abstract

To examine the possibility that the bone marrow cells of BALB/c genotype interfere with the development of insulitis and diabetes in NOD mice, we transplanted BALB/c bone marrow cells mixed with NOD bone marrow cells into NOD mice. The [(NOD + BALB/c) → NOD] chimeras developed insulitis and diabetes, indicating that BALB/c bone marrow cells do not interfere with the development of the disease in NOD mice. Surprisingly, these mice have been reconstituted with only NOD hematolymphoid cells. When the pancreatic tissues from newborn NOD and BALB/c mice were grafted into [(NOD + BALB/c) → NOD] chimeras, the BALB/c pancreatic tissues were rejected, whereas the NOD graft showed insulitis. Furthermore, the spleen cells of the chimeras showed responsiveness to BALB/c spleen cells in mixed lymphocyte reaction and generated cytotoxic T lymphocytes specific for the H-2^d and third party targets. These findings indicate that the hematolymphoid cells (including hemopoietic stem cells) of NOD mice are more resilient than those of normal BALB/c mice, and that insulin-dependent diabetes mellitus will recur after bone marrow transplantation unless the hematolymphoid cells of NOD mice are completely destroyed by irradiation.

Introduction

NOD mice spontaneously develop insulitis followed by diabetes. T cells infiltrate the pancreatic islets and destroy insulin-producing beta cells (1–5). In addition to environmental factors, the development of the disease is regulated by multiple genetic factors. In NOD mice, nine or more suscepti-

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Abbreviations: *BM* = bone marrow; *BMT* = bone marrow transplantation; *MLR* = mixed lymphocyte reaction; *CTLs* = cytotoxic T lymphocytes; *GTT* = glucose tolerance test; *MHC* = major histocompatibility complex; *HSCs* = hemopoietic stem cells

bility genes are proposed (6–12). The susceptibility genes should express their effect through particular cells and/or tissues, and the events controlled by these genes should emerge sequentially, resulting in the destruction of islets and diabetes.

The development of insulitis and diabetes has been demonstrated in C3H/HeN, (BALB/c × B6)F1, (NOD × B10)F1, and (NOD × NON)F1 mice following reconstitution with BALB/c → NOD bone marrow (BM) cells (13–16). Conversely, we have demonstrated that neither insulitis nor diabetes develop in the [BALB/c → NOD] mice (17, 18). Therefore, BM cells of NOD genotype play a crucial role; the effects of some susceptibility genes are expressed through BM cells. However, NOD BM cells do not induce the disease in the [NOD → BALB/c] chimeras by 8 months (19), but induce the disease more than one year after BMT in [NOD → C3H] mice (13). These observations suggest that radioresistant BM or other cells of normal mouse genotype interfere with the development of insulitis and diabetes by NOD hematolymphoid cells.

In the present study, we examine whether BALB/c BM cells suppress the development of insulitogenic and diabetogenic T cells from NOD BM cells in the NOD environment, and show that BALB/c BM cells are unable to suppress the development of insulitis and diabetes in the [(NOD + BALB/c) → NOD] chimeras.

Materials and Methods

Mice

NOD (K^d, $I\text{-}A^{g7}$, D^b) mice were bred and maintained under specific pathogen-free conditions in our animal facility. BALB/c (K^d, $I\text{-}A^d$, D^d) and C57BL/6 (K^b, $I\text{-}A^b$, D^b) mice were purchased from CLEA Japan, Inc. (Osaka). In all experiments, 6- to 12-week old female mice were used except for the neonatal donors of pancreas grafts.

Bone marrow transplantation (BMT) and pancreas transplantation

Donor BM cells collected from femurs of NOD and BALB/c mice were treated separately with anti-Thy 1.2 monoclonal antibody (F7D5, Olac, Bicester, England) plus rabbit complement at 37 °C for 40 min to eliminate T cells, as previously described (19). Recipient NOD mice were lethally irradiated (9.5 Gy, ^{60}Co) and injected intravenously with 2×10^7 BM cells in which NOD and BALB/c cells were mixed in the ratio of 1 : 1, 3 : 1 or 10 : 1. Pancreatic tissues from newborn NOD and BALB/c mice were grafted under the renal capsules as previously described (19).

Cytotoxicity test

Spleen cells were suspended in TC-199 (GIBCO, Grand Island, NY, USA) with 5 % fetal calf serum (FCS; Hyclone Laboratories, Logan, UT, USA). Cell concentration was adjusted to 5×10^6 cells per ml, and cells were divided into two aliquots of 50 µl. The cells were incubated with 50 µl of a 1 : 25 dilution of anti-H-$2K^d$, anti-H-$2D^b$ antibody for 30 min at 4 °C and then washed once and resuspended in 100 µl of a 1 : 10 dilution of rabbit complement previously absorbed with mouse spleen cells. After a 30-min incubation at

37 °C, the viability of the cells was determined by trypan blue dye-exclusion. The counts were converted to a cytotoxicity index by the formula:

$$CI = \frac{\%\ V\ cells\ (C\ alone) - \%\ V\ cells\ (Ab + C)}{\%\ V\ cells\ (C\ alone)} \times 100$$

where *CI* represents cytotoxicity index; *V cells* = viable cells, *C* = complement, and *Ab* = antibody.

Flow cytometric analyses

Spleen cells were suspended in RPMI 1640 and stained with FITC-conjugated anti-H-2 monoclonal antibodies anti-H-2K^b (030-11F), anti-H-2K^d (030-21F), anti-H-2D^b (030-14F), anti-H-2D^d (030-20 F), Meiji Institute of Health Science, Odawara, Japan. Cells were fixed in 2 % paraformaldehyde solution. Flow cytometric analyses were performed using a FACScan or FACStar. Peripheral blood was collected from the tail veins in a heparinized tube, washed, layered on Lymphocyte Separation Solution (JIMRO, Gunma, Japan), and centrifuged at 2,000 rpm for 20 min. Mononuclear cells at the interface were collected, washed, stained, and analyzed.

Mixed lymphocyte reaction (MLR)

The MLR was estimated by counting the incorporation of ^{3}H-TdR into cells. Triplicate cultures were set up in 96-well round-bottomed microtiter plates (25850, Corning Glass Works, Corning, NY, USA). Each well contained 5×10^5 responder cells and 2.5×10^5 stimulator cells (25 Gy-irradiated) in a total volume of 200 µl of RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10 % heat-inactivated fetal calf serum, and 5×10^{-5} M 2-mercaptoethanol. The cultures were incubated for 96 h in a humidified atmosphere of 5 % CO_2 in air at 37 °C. During the last 4 h of culture, 0.5 µCi of ^{3}H-TdR was added. The uptake of ^{3}H-TdR was counted using a liquid scintillation counter.

Generation of cytotoxic T lymphocytes (CTLs)

Responder spleen cells (7.5×10^6) and 20 Gy-irradiated stimulator spleen cells (2.5×10^6) were cocultured in a 24-well plate (Flow Laboratories, Inc., Virginia, USA), in culture medium. Five days later, the cells were collected and their cytotoxic activity was determined by the ^{51}Cr-release assay. P815 (H-2^d), EL-4 (H-2^b), and X5563 (H-2^k) target cells were labeled with ^{51}Cr and adjusted to 5×10^5/ml. Effector cells (5×10^6/ml) were cultured with equal volumes of target cells in a round-bottom microtiter plate for 4 h. The radioactivity of 100 µl of supernatants was evaluated using a gamma-counter. Percent-specific lysis (%SL) was calculated as follows:

$$\%SL = \frac{(\text{Experimental } ^{51}\text{Cr release}) - (\text{Spontaneous } ^{51}\text{Cr release})}{(\text{Maximum } ^{51}\text{Cr release}) - (\text{Spontaneous } ^{51}\text{Cr release})} \times 100$$

Glucose tolerance test (GTT)

Mice were kept fasting for 16 h before GTT. Peripheral blood was collected from the orbital plexus into heparinized glass tubes before and 30, 60, and 120 min after intraperitoneal injection of 1 g/kg glucose. The blood sugar concentration was measured by test tapes (Lilly, Indianapolis, USA) and a dextrometer. The mice with more than

150 mg/dl blood sugar before the injection of glucose and more than 200 mg/dl at 30 and 60 min after the injection were assumed to be diabetic.

Histopathological studies

Major organs including the grafts were removed for histological study. Organs were fixed with 10 % formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (H-E). The degree of lymphocyte infiltration of the pancreatic tissues was scored according to the criteria of WICKER et al. (8). Score 0 indicates no inflammatory cells in the pancreas; 1, mononuclear cells in the periductal and/or perivascular area; 2, small numbers of islet-associated mononuclear cells at the islet periphery; 3, moderate to severe infiltration of mononuclear cells in the islet with manifestation of beta-cell destruction. Depending on our previous observations (13, 20), fibrotic changes in the grafts without pancreatic tissues such as islets or ducts were assumed to show rejection.

Results

Development of insulitis and diabetes in mixed allogeneic chimeric mice

Seven female NOD mice were reconstituted with T cell-depleted mixed BM cells of female NOD and BALB/c mice in a one-to-one ratio. The chimeric mice were monitored monthly by GTT. Four mice developed diabetes

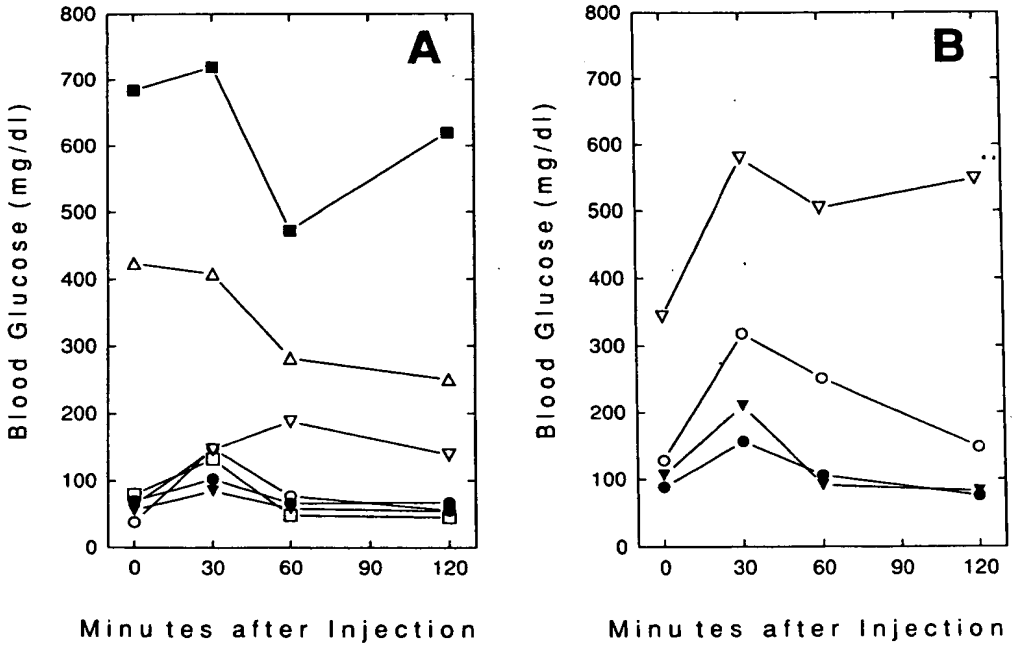

Figure 1. Glucose tolerance test (GTT) in [(NOD + BALB/c) → NOD] chimeric mice. GTT was carried out 4 weeks (A) and 16 weeks (B) after BMT. Symbols are shared by the individual mice in A and B. Two mice were diabetic at 4 weeks, and another two became diabetic by 16 weeks. Four out of 7 mice at 4 weeks and 2 out of 2 at 16 weeks show a normal response on GTT.

Table 1. Insulitis and diabetes in [(NOD + BALB/c) → NOD] mixed bone marrow chimeras.[a]

Animal no.	Pancreas histology					Development of diabetes	Onset of diabetes (mo)
	Number of islets observed	Histology Score[b]					
		0	1	2	3		
		%[d]					
1	no islet	nd[e]	nd	nd	nd	+	<4
2	31	22	8	0	3	–	
3	19	0	5	16	79	+	<1
4	12	42	42	0	16	–	
5	8	0	0	38	62	+	<3
6	15	0	7	7	86	–	
7	7	0	0	43	57	+	<1

[a] The mixture of T cell-depleted bone marrow cells from 6-week old NOD and 8-week old BALB/c mice was transferred into lethally irradiated 2.5-month old NOD mice. The recipient mice were observed for 4 months after bone marrow transplantation.
[b] For insulitis scores, see «Materials and Methods».
[c] Development of diabetes was monitored by monthly carrying out glucose tolerance tests after BMT.
[d] Percentages of islets were evaluated under the scores.
[e] Not determined due to absence of islets.

within 16 weeks after BMT (Fig. 1), and 2 of these 4 showed diabetic patterns on GTT as early as 4 weeks after BMT (Fig. 1A). All diabetic mice showed polydipsia, polyuria, and loss of body weight. In all four mice, histological examinations revealed the typical insulitis observed in NOD mice (Table 1). Immunohistochemical studies showed that insulin-producing beta cells had been selectively destroyed (Fig. 2).

Another six female [(NOD + BALB/c) → NOD) chimeric mice were grafted with both newborn NOD and BALB/c pancreatic tissues two months after BMT. Two weeks later, these mice were sacrificed and examined for insulitis (Table 2). In all six chimeric mice, insulitis was observed in the host and/or grafted NOD pancreatic tissues (Fig. 3a), whereas the grafts of BALB/c pancreatic tissue was replaced by fibrotic tissue (Fig. 3b), indicating that the BALB/c pancreatic tissues were rejected.

Analyses of chimerism in [(NOD + BALB/c) → NOD] mice

Mice were examined for chimerism using a cytotoxicity test or a flow cytometer. Two weeks after BMT, peripheral blood mononuclear cells consisted of both BALB/c- and NOD-derived cells (Table 3). However, by

Figure 2. Selective destruction of beta cells in a [(NOD + BALB/c) → NOD] mouse. Insulin-producing cells (a) are not observed, although glucagon-producing cells (b) or somatostatin-producing cells (c) are present.

Table 2. Insulitis in [(NOD + BALB/c) → NOD] mice grafted with newborn pancreatic tissues from NOD and BALB/c mice.[a]

Animal no.	Pancreas graft						Own pancreas				
	NOD					BALB/c					
	Number of islets observed	Histology score[b]					Number of islets observed	Histology score[b]			
		0	1	2	3			0	1	2	3
		%						%			
1	5	0	40	60	0	fibrosis	20	25	30	35	10
2	10	50	20	10	20	fibrosis	29	48	28	14	10
3	no islet	nd	nd	nd	nd	fibrosis	22	50	27	14	9
4	21	14	62	19	5	fibrosis	54	67	24	9	0
5	4	0	75	25	0	fibrosis	29	76	21	3	0
6	10	50	20	10	20	fibrosis	47	55	30	11	4

[a] The mixture of T cell-depleted bone marrow cells from 6-week old NOD and 6-week old BALB/c mice was transferred into lethally irradiated 6-week old NOD mice. Two months later, neonatal pancreatic tissues from NOD and BALB/c mice were transplated to the [(NOD + BALB/c) → NOD] mice. Two weeks later, the grafts and recipients' own pancreatic tissues were examined.
[b] See footnote of Table 1.

3 weeks, NOD-derived cells had become dominant (Fig. 4). Similar findings were obtained even when the donor BM cells from BALB/c mice increased to three times (15×10^6 BALB/c plus 5×10^6 NOD) or ten times (20×10^6 BALB/c plus 2×10^6 NOD) those of the donor NOD mice (Table 3). These findings indicate that the BALB/c-derived cells decrease in number and finally disappear in the [(NOD + BALB/c) → NOD] chimeric mice.

Analyses of tolerance in [(NOD + BALB/c) → NOD] mice

The degree of tolerance of 4 [(NOD + BALB/c) → NOD] chimeric mice was analyzed at 16 weeks after BMT. As shown in Figure 5, spleen cells from chimeric mice clearly showed responses comparable to those of naive NOD mice against BALB/c ($H\text{-}2^d$) stimulator cells. The [(NOD + BALB/c) → NOD] spleen cells did not respond to the NOD stimulator cells (data not shown). Furthermore, the spleen cells of 3 [(NOD + BALB/c) → NOD] mice generated CTLs specific for the $H\text{-}2^d$ target cells (Table 4).

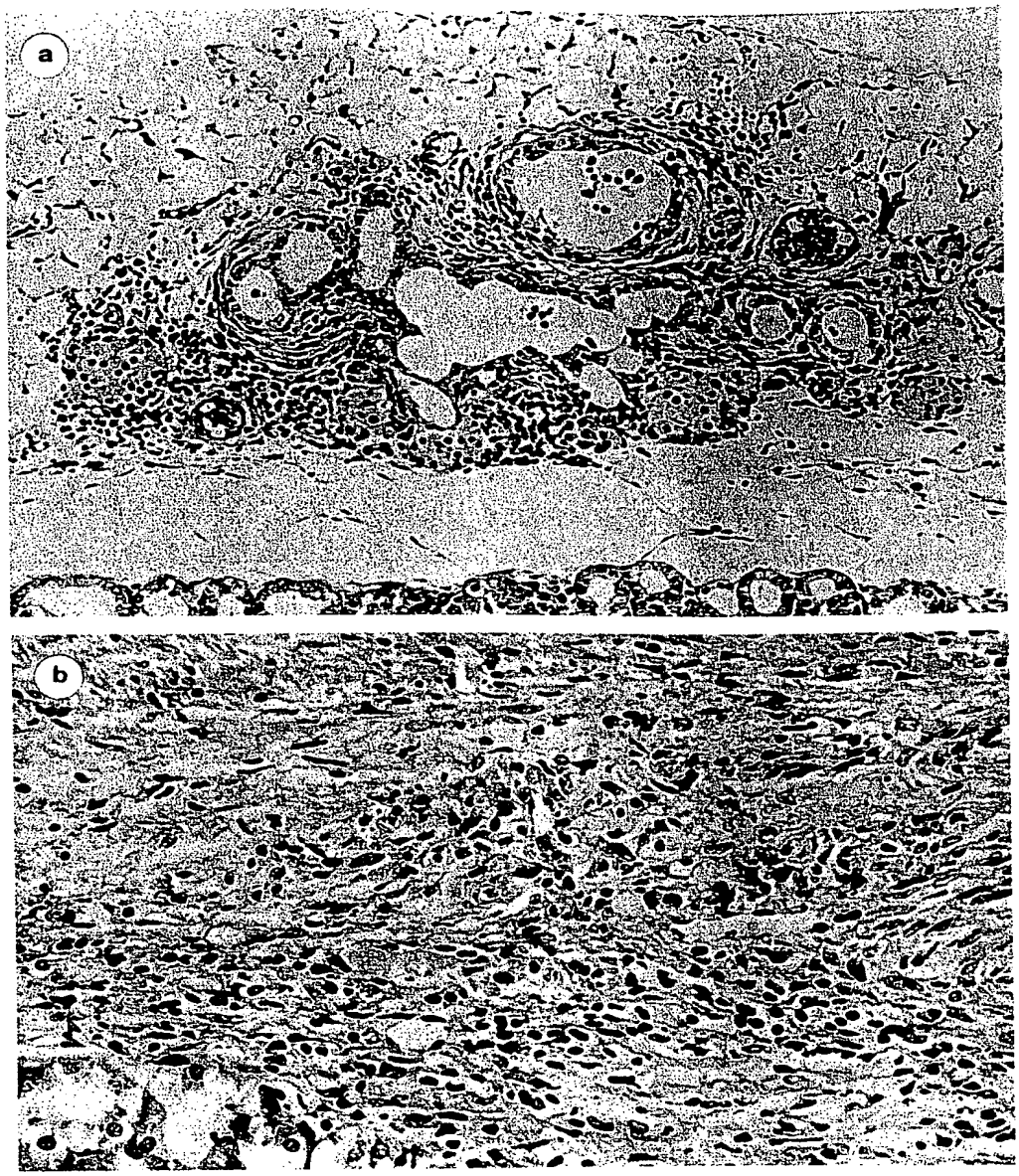

Figure 3. Histology of pancreatic grafts under the capsule of the kidney in a [(NOD + BALB/c) → NOD] chimera 2 weeks after transplantation. NOD pancreata are engrafted with infiltration of lymphocytes around the islets, leaving pancreatic ducts intact (**a**). BALB/c grafts have become fibrotic and devoid of pancreatic islets and ducts (**b**).

Discussion

We attempted to establish allogeneic mixed chimerism using T cell-depleted syngeneic (NOD) and allogeneic (BALB/c) bone marrow cells. All the recipient mice, however, showed insulitis, and 4 out of 7 mice developed

Table 3. Analyses of chimerism in [(NOD + BALB/c) → NOD] mice.

Ratio (BALB/c:NOD)	Phenotype	After BMT		
		2 weeks	3 weeks	2 months
		% (no. of mice examined)		
1 : 1	H–2K^d	68.3 (1)	94.1 (1)	98.9 (1)
	H–2D^b	35.8 (1)	88.0 (5)	88.7 (1)
	H–2D^d	ND	6.1 (5)	ND
3 : 1	H–2K^d	ND	93.9 (2)	97.0 (1)
	H–2D^b	ND	84.0 (2)	96.8 (3)
	H–2D^d	ND	18.8 (2)	5.6 (3)
10 : 1	H–2K^d	ND	93.4 (1)	95.0 (1)
	H–2D^b	ND	80.6 (1)	95.9 (4)
	H–2D^d	ND	ND	3.6 (4)

ND = not determined.

NOD mice (K^d, I–A^{g7}, D^b) were lethally irradiated and then reconstituted with T cell-depleted BALB/c (K^d, I–A^d, D^d) and NOD bone marrow cells mixed at 1 : 1, 3 : 1, and 10 : 1 ratios. The percentages of phenotypes in the mixed chimeras were examined 2 weeks, 3 weeks, and 2 months after BMT.

overt diabetes. The group of SACHS et al. (21–25) reported that the reconstruction of B10 mice with T cell-depleted bone marrow cells from syngeneic B10 and allogeneic B10.D2 mice resulted in mixed allogeneic chimerism; graft failure occurred only when the syngeneic donor bone marrow cells had not been treated with anti-T cells antibodies (particularly Lyt-2 antibody). We have confirmed their findings using normal mouse

Table 4. Generation of cytotoxic T lymphocytes from spleen cells of [(NOD + BALB/c) → NOD] mice.[a]

Mouse	% Specific lysis (E/T = 40/1)		
	P815 (H–2^d)	EL–4 (H–2^b)	X5563 (H–2^k)
BALB/c (H–2^d)	3	46	51
C57BL/6 (H–2^b)	75	0	36
C3H/HeN (H–2^k)	80	44	0
[(NOD + BALB/c) → NOD]			
No. 1	68	12	16
No. 2	47	30	22
No. 3	33	32	24

[a] Spontaneous release and maximum releases of P815 were 424 and 2,194, of EL–4 were 929 and 3,279, and of X5563 were 679 and 3,957 cpm, respectively.

combinations such as [(B6 + C3H)→ B6]: [(B6 + C3H)→ B6] chimeric mice do not reject C3H pancreas, although host (B6)-derived hemopoietic cells gradually increase (data not shown). In our experiments, however, the allogeneic normal BALB/c bone marrow cells in the recipient NOD mice were eventually eliminated after BMT, and the engrafted pancreatic tissues were also rejected, although we used T cell-depleted syngeneic and allogeneic bone marrow cells.

In this experiment, the failure in mixed allogeneic chimerism in [(NOD-+BALB/c)→ NOD] mice may be due to the development of abnormal lymphocytes from stem cells of NOD mice; these lymphocytes are neither tolerant of NOD beta cells nor of BALB/c-type major histocompatibility complex (MHC) determinants, as shown in Figure 5 and Table 4. Another possibility is that the abnormal hematopoietic environment of NOD mice prevents the implantation (or growth) of BALB/c mice bone marrow cells. However, this is unlikely, since we have previously demonstrated that BMT from BALB/c to NOD mice prevents insulitis and diabetes (17). The failure to implant BALB/c bone marrow cells is not related to the quantity of donor bone marrow cells, since the transplantation of ten times the number of cells also led to the elimination of allogeneic (BALB/c) cells (Table 3). It is therefore likely that the hematolymphoid cells of NOD mice are tougher than those of normal BALB/c mice. We have recently demonstrated by BMT between normal and autoimmune-prone mice that both systemic and organ-specific autoimmune diseases are stem cell disorders (13, 26). To examine the differences between normal and abnormal hemopoietic stem cells (HSCs), we have been attempting to establish a method for the purification of HSCs (27, 28) and have finally succeeded (29). Using this method, we have found that the HSCs of autoimmune-prone mice are more resilient than those of normal mice both *in vivo* and *in vitro*. Although the HSCs of autoimmune-prone mice can proliferate and differentiate in the allogeneic environment without the help of MHC-matched stromal cells and/or other cells, normal HSCs cannot survive in the allogeneic environment without the help of MHC-matched stromal cells and other cells (KAWAMURA, M., et al., submitted for publication). In addition, we have also found that the HSCs from autoimmune-prone mice have the capacity to proliferate and differentiate *in vitro* by interaction with even MHC-mismatched stromal cells (HISHA, H., et al., manuscript in preparation). Regarding MHC restriction between HSCs and stromal cells, we have very recently demonstrated the requirement of donor-derived stromal cells for successful allogeneic BMT (30). From these findings, it is conceivable that [(BALB/c + NOD)→ NOD] chimeras develop insulitis and diabetes. We therefore propose that allogeneic BMT (but not mixed allogeneic or autologous BMT) should be used for the treatment of autoimmune diseases.

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